EVALUATION OF A PARTIALLY DE-OILED MICROALGAE PRODUCT IN NURSERY PIG DIETS

A THESIS
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

ADVISER: MILENA SAQUI-SALCES

AUGUST 2019

Acknowledgements

Without a doubt, this has been one of the most challenging experiences of my life. There were many times throughout my masters when giving up seemed to be the easiest route. However, I was extremely fortunate to be surrounded by people who would not allow me to do so and enabled me to see my true potential, ultimately pushing me to accomplish this immense goal of mine. To these people, I want to say thank you from the bottom of my heart for believing in me, for being there when I needed you the most, and for creating an environment in which dedication and compassion thrived.

To my advisor, Dr. Milena Saqui-Salces: thank you for providing me with words of advice that allowed me to always see the bigger picture, which grounded me during my times of stress and anxiety. Thank you for treating me as a person rather than simply a student, and for understanding that life sometimes leads you down challenging paths, requiring periods of reflection and maturity. I can say with confidence that I am not the same person that I was when I started my master's, and I have you to thank for a large proportion of that growth. For that, I am extremely grateful.

To my committee members: Dr. Gerald Shurson, thank you for dedicating your life's work to this industry, and for constantly working to provide a better future for your students to give them a means to gain an education and thrive in a professional setting. Dr. Pedro Urriola: your work ethic and dedication to this industry and to your students truly knows no bounds. I cannot thank you enough for being present at any time or day to work through any question or problem that I ever had. Dr. Chi Chen: thank you for

always showing me a unique way of addressing a challenge or question. I have learned a great deal from your teaching methods, and I thank you for inspiring me to be a better student.

To Dr. Lee Johnston and to the team at the West Central Research and Outreach Center, thank you so much for all your help and support with designing and completing the feeding trial for my experiment. You showed me what hard work and dedication truly looks like, and I will always remember that experience.

To Dr. Marshall Stern, I cannot thank you enough for all that you have done for me since I met you in the start of my academic career as an undergraduate and until now as a master's student. I always had the best conversations with you that were filled with both great advice and humor.

To Dr. Christopher Faulk, and Dr. Laura Mauro, thank you for supporting me and for being genuinely good people. I always looked forward to talking with each of you throughout the day.

To Allison Kierth, thank you for your dedication and compassion that you have for this department and its students and for your support through my challenging periods of my master's program.

To all the graduate students and my friends: Mickie Trudeau, Leonard Fung, Marta Ferrandis Vila, Edward Yang, Yuantai Yung, Mathia Colwell, Amanda Borchers, Ramya Lekha Medida, Julia Holen, Zhimin Huang, Zhikai Zeng, Jinlong Zhu. Together, we have experienced a great deal of both stress and joy. I would not have survived this program without each and every one of you, and I will never forget the experiences that we shared. You all inspire me to be a better person.

Dedication

This thesis is dedicated to my family: Mom, Dad, Katie (Trace, Sophia, Emmy, Isabel), Danny (Sara, Nora, Jerrie), Pat (Sara), Glenn, Auntie, Siti, Baka, Nanny and Papa. Thank you for showing me what love and family really means. For teaching me how to be a good person inside and out, no matter the time or place. For giving me the means to succeed and putting our family first before yourselves in every aspect of your lives. You are all my greatest inspiration, and this accomplishment is just as much yours as it is mine. And to my incredible girlfriend, Claire. You have been through this with me every step of the way, supporting me no matter how unconfident I was in myself or in this process. You gave me the strength to keep going and you continue to bring out the best in me. I am so excited to start this new chapter of our lives together. To the Gillick family, thank you for all your continued support throughout this challenging time, and for cheering for me. I feel extremely fortunate and blessed to have all of you in my life.

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Abbreviations

ALG	Algae meal
CDC	Center for Disease Control and Prevention
CO2e	CO_2 equivalents
DHA	Docosahexaenoic acid
DFA	Defatted diatom microalgae
DW	Dry weight
EPA	Eicosapentaenoic acid
FDA	Food and Drug Administration
MA	Marine algae product
MAE	Microalgae extract
ME	Metabolizable energy
NRC	National research council
NSP	Non-starch polysaccharides
PUFA	Polyunsaturated fatty acids
SCFA	Short chain fatty acids
SID	Standardized ileal digestible
PUFA	Polyunsaturated fatty acids
TAG	Triglycerides
USDA	United States Department of Agriculture
SCFA	Short chain fatty acids

Chapter 1. Introduction and literature review

1.1 Introduction

The world's population is expected to increase from 7 billion to 9 billion by the year 2050. Consequently, production of corn and soybeans, two of the most common crops used in developed countries to feed livestock will need to increase by 80 and 140% by 2050 to provide feed security for both animals and humans. However, arable land available to grow those crops is rapidly declining (Bruinsma, 2009). For swine producers, production of feed represents 65-75% of total costs. In addition, the demand for pork is high, as it represents the largest percent of all meat consumed in the world (~37%) (McGlone, 2013). According to the United States Department of Agriculture (USDA), there were 73.2 million pigs on U.S. farms in 2017 alone, the most seen since 1943, with 11.8 billion kg of pork produced, with pork exports totaling 20.2 billion kg (Waters & Hirtzer, 2018). The USDA estimates that in 2019, pork production will be 3.8% above the level it was in 2014, which is similar to the growth of world population.

The primary focus in the swine industry now is to find and incorporate alternative feed ingredients to offset cost and demand for corn and soybean, to ultimately provide feed security for future generations. Currently, microalgae are being studied for their potential as alternative feed ingredients for the swine industry.

1.2 Definition and history of microalgae

Algae are defined as any organism that contains chlorophyll *a* and a thallus that is not differentiated into roots, stems, or leaves. Microalgae, specifically, are prokaryotic or

eukaryotic microorganisms which constitute over 100,000 species (Lum et al., 2013). Microalgae can be found in marine or freshwater environments and are classified by type of pigment, chemical nature of storage products, and cell wall characteristics (Becker et al., 2004). Common classes of microalgae are *Cyanophyceae* (blue-green algae), *Chlorophyceae* (green algae), *Bacillariophyceae,* and *Chrysophyceae* (golden algae) (Carlsson et al., 2007) (Table 1.1). Microalgae are also classified by their means of acquiring energy or cultivation techniques and can be separated into autotrophic and heterotrophic groups. Autotrophic microalgae acquire energy by converting solar energy into chemical energy via photosynthesis, while heterotrophic microalgae obtain their energy from glucose or external, organic carbon sources (Carlsson et al., 2007). The majority of microalgae are autotrophic.

Research on microalgae as a feed ingredient first began in the early 1950's, as the demand for alternative protein sources increased due to concerns of food security with a rising world population (Becker et al., 2004). In 1960, the first large scale production systems of microalgae began in Japan, with cultivation of *Chlorella,* one of the most commonly known microalgae species based on its high protein content at 60% on a DM basis (Borowitzka et al., 1999). Within 10 years, microalgae production increased, with Asia producing more than 1,000 kg of microalgae per month for protein use for human consumption.

Microalgae were also researched for their potential as a renewable energy source, as they are able to sequester CO_2 via photosynthesis, which in turn could be used to produce hydrogen and methane gas (Benemann 2013)**.** Today, research on microalgae is focused on their potential use as both a sustainable source of energy (biofuel) and

dietary ingredient for human and animals. The global algae products market reached $3.40 billion in 2017, and is expected to rise to $6.09 billion by 2026, representing a compound annual growth rate of 6.7%. The focus of this thesis will be on microalgae researched as alternative feed ingredients to potentially solve the challenges within the swine industry.

1.3 Microalgae potential use in swine industry

1.3.1 Land, water, and carbon footprint of pork industry

It is estimated that agricultural production, including cropland and grassland, occupies 43% of Earth's ice-free surface (Foley et al., 2011). An estimated total amount of land used by the pork industry in the U.S. was 6,000,000 ha/yr, with 96% of that used toward the production of feed. The U.S. pork industry uses approximately two trillion L of water/yr for crop production for swine rations accounting for 83-93% of the pork supply chain water footprint. The estimation for the carbon footprint of producing a 113 g serving of pork was 1.12 kg CO_2 equivalents (CO_2e), with 52.5% contributed from the live production of pigs from the nursery to finish stage (Thoma et al., 2011; Thoma et al., 2011; Matlock et al., 2011). The production of feed is the greatest contributor to the overall environmental impact of the U.S. pork industry, with corn requiring the most resources, with 0.9 time of land occupied (m^2a), 0.04 m^3 of water, and 0.3 kg CO_2e/1 kg of dry feed (MacLeod et al., 2013; Liedke et al., 2016). Large scale cultivation of microalgae could improve sustainability of the swine industry, as they do not require arable land for growth, and therefore do not compete with traditional feed crops like corn and soybean. A recent study identified 90,000 potential

sites in the U.S. for microalgae cultivation, totaling 5.5% of U.S land mass (Langholtz et al., 2016).

1.3.1.1 Microalgae use for sustainability

a.) Open cultivation systems

Typical large-scale production systems of microalgae are called open-cultivation systems, otherwise known as raceway ponds which utilize a paddle-wheel to drive current for microalgae cultivation, with a primary goal of capturing maximum amounts of light (Buchanan et al., 2013). Although relatively lower in cost of construction (~ $100,000/ha) compared with other cultivation systems, raceway ponds produce less biomass, on average 40 $g/m^2/d$ (Carlsson et al., 2007; Acién et al., 2017). As open-air systems, raceway ponds are commonly subject to contamination and nutrient competition by other algae, protozoa and pressure from an uncontrollable environment, especially due to weather. Based on the need for consistent sunlight for growth, raceway ponds are primarily limited to warm climates. To mitigate issues involving contamination, raceway ponds are kept at high salinity and alkalinity, consequently creating additional limitations on what species can successfully grow in these systems. Common species grown in raceway ponds include *Chlorella, Spirulina,* and *Dunaliella* (Buchanan et al., 2013).

b.) Closed cultivation systems

Closed cultivation systems, alternatively named photobioreactors, can mitigate limitations such as contamination and competition. Photobioreactors use tubes to pump circulating microalgae culture and allow for a more controlled environment (i.e.

temperature, shelter from weather) and less contamination by surrounding algae (Van Iersel et al., 2010). In addition, photobioreactors require less land to operate (~ 10 ha per unit) and produce more biomass than raceway ponds (Slade et al., 2013). Closed cultivation systems that utilize heterotrophic microalgae produce approximately 5.8 g/ L/ d of biomass compared to 0.4 g/L/ d of biomass in raceway systems that require light to grow (Van Iersel et al., 2010). However, photobioreactors have considerably higher capital costs compared to raceway ponds, at approximately $1,000,000 / ha (Carlsson et al., 2007).

c.) Biofuel industry

Biofuels are defined as renewable fuels derived from biologically based feedstocks and are researched due to increased demand for renewable and sustainable sources of energy (Abomohra et al., 2016). Biodiesel, specifically, is defined as the fatty acid methyl esters derived from the transesterification of renewable oil feedstocks using alcohol and acid or base catalysts. In the U.S., soybeans are the most common source of feedstock for biodiesel production (Chisti et al., 2007). However, with the demand for soybeans set to increase in the future and a reduction in arable land for farming, microalgae are researched as an alternative source for biodiesel production based on the high lipid content in some species (Mata et al., 2010). The oil content and biodiesel productivity from microalgae is significantly higher than soybean. Soybeans produce an average of 636 L oil/ha/yr, 562 kg biodiesel/ha/yr, and use 18 m^2/yr of land/kg biodiesel (Mata et al., 2010). Comparatively, a microalgae source that is considered "low" in oil content produces 58,700 L oil/ha/yr, and produces 51,927 kg

biodiesel/ha/yr, while at the same time uses a considerable less amount of land at 0.2 m^2/yr/kg biodiesel compared to soybeans (Mata et al., 2010).

d.) Microalgae byproducts

A source of microalgae for animal production systems, which is a byproduct of the biofuel industry, is called defatted microalgae. Briefly, the lipids within microalgae are extracted by dewatering the microalgae, and subsequently sampled through solvent extraction. The leftover biomass of microalgae after this lipid extraction is then called "defatted" (Lum et al., 2013). De-oiled microalgae is also a byproduct derived from heterotrophic microalgae grown in large fermentation tanks. These heterotrophic microalgae are then provided an external carbon source, such as sugarcane to grow. The microalgae within the tanks are sampled and a portion of the oil is mechanically removed, leaving a partially de-oiled microalgae byproduct (Solazyme, 2017). As these are byproducts of large scale cultivation, no additional natural resources are required for their growth, thus providing additional outlets for sustainability.

1.3.1.2 Uses of microalgae

a.) Wastewater treatment

Microalgae can take up excess metals from their environment with a two-phase process: adsorption and absorption. Adsorption occurs via extracellular associated material, such as polysaccharides and mucilage, as well as components of the cell wall like carboxy and hydroxy groups. Absorption involves the accumulation of heavy metals inside the cell, leading toward detoxification via the formation of metal binding peptides and proteins like metallothioneins and phytochelatins (Becker et al., 2004). Research is now focused on using microalgae for treating waste from common food

production systems, such as the dairy and meat industry, which in turn uses the nitrogen and phosphorus rich effluent leftover to grow new batches of microalgae for use in animal feed (Buchanan et al., 2013). Kebede-Westhead et al., (2006) utilized freshwater microalgae to treat waste from swine. With a loading rate of 0.40 L/m/d of waste, results showed that 9.4 g dry weight (DW)/m/d of algae biomass were harvestable, and 95% of nitrogen (N) and 77% of phosphorus (P), were removed by the algae. The same freshwater microalgae specie was used to treat anaerobically digested dairy manure effluent, with a loading rate of 9 L/m/d of waste. Kebede-Westhead et al., (2006) observed a harvestable biomass of 17.7 g DW/m/d, and a removal efficiency of N and P of 68% and 73%, respectively. Differences in harvestable algal biomass between swine and dairy wastewater was due to higher loading rates of nutrients such as nitrogen and phosphorus in dairy waste.

To provide a more stable environment for microalgae to grow, mixtures of wastewater are now utilized. Lu et al., (2015) investigated potential biomass yields of *Chlorella* sp. grown on mixed sources of meat wastewater, compared to biomass yields of microalgae grown on non-mixed sources of wastewater. The greatest biomass yield obtained using a mixed source of wastewater was 1.5 g/L. Comparatively, Woertz et al., (2009) for example, showed an average yield of algal biomass of 0.5 g/L grown on one source of dairy wastewater.. Microalgae grown from treating wastewater represents a highly sustainable system of production, by recycling waste for nutrients and limiting the utilization of natural resources.

1.3.2 Antibiotic use

Antibiotics have been used throughout the swine industry for both therapeutic and sub therapeutic effects. Therapeutic inclusion with the aim of treating disease and sub-therapeutic inclusion to increase growth performance. The presence of bacteria resistant to common antibiotics such as methicillin and tetracycline has been documented in U.S. swine production systems (David and Daum, 2010; Smith et al., 2013; Ferguson et al., 2016). Furthermore, the Center for Disease Control and Prevention (CDC) estimates that every year more than 400,000 people in the U.S. suffer from infections caused by drug-resistant bacteria. Due to this threat, the Food and Drug Administration (FDA) developed the veterinary feed directive guidance in 2017 approving the use of medically important antibiotics in animals only for the treatment, control and prevention of disease, and prohibiting the use of antibiotics as growth promoters. Although the use of antibiotics as growth promoters has ceased, the most recent FDA report on medically important antibiotics used in food animal production estimated that 6,000,000 kg of antibiotics were sold in 2017. The report also noted that 36% of those medically important antibiotics were used in swine production for therapeutic purposes, ranking second under cattle for most antibiotics used in 2017 (Food and Drug Administration, 2017).

Microalgae have been researched for their health promoting attributes with the potential to mimic the effects of therapeutic antibiotics, to continue in minimizing the use of medically important antibiotics in swine production, especially during the weaning period. It has been estimated that 80% of antibiotics used in swine production

are used through oral administration in pigs less than ten weeks of age (Lekagul et al., 2019).

1.3.2.1 Health promoting properties of microalgae

Studies have shown that fermentable and digestion resistant carbohydrates from microalgae can act as prebiotics, and potentially lead to mucosal development and restoration in weaned piglets (De Jesus Raposo et al., 2016; Furbeyre et al., 2016). These effects are described in further detail in section 1.4.4. Polysaccharides synthesized by microalgae are also studied for their antiviral characteristics. *Porphyridium cruentum* is studied for its sulphated polysaccharides which specifically inhibit the activity of viruses by competitive blocking the interaction of the virus with the host cell receptor (Pradhan et al., 2014).

The fatty acids derived from microalgae are researched for their antibacterial properties. Specifically, unsaturated and saturated long chain fatty acids from microalgae have been shown to induce lysis of bacterial protoplasts and provide population defense for the host. For example, when a microalgae cell is damaged by a pathogen, that cell will release fatty acids that can act on the pathogens in the surrounding area to decrease their population and prevent further pathogen dissemination **(**Desbois et al., 2012). *Chlorella* was the first microalgae species researched for its antibacterial effects by Pratt et al., (1944), who with a mixture of fatty acids called *Chlorellin* were successful in inhibiting the population of both Gram positive and Gram negative bacteria (Spruijt et al., 2016). Fatty acids from *Spirulina platensis* have also been proven to have antibacterial effects against Gram positive

bacteria like *Streptococcus sp.* as well as Gram negative bacteria like *E. coli* (Priyadarshani et al., 2012).

Carotenoids especially have been attributed to the antioxidant properties of microalgae. Common carotenoids from microalgae that are utilized for their antioxidant properties include β carotene, lutein, and astaxanthin. Astaxanthin acts as a strong antioxidant by reducing and stabilizing free radicals, and is considered to be approximately 65 times more powerful than common antioxidants such as vitamin C and β carotene (Shah et al., 2016).

1.3.2.2 Toxicity of microalgae

Nutritional toxicity from microalgae is a concern due to microalgae's ability to accumulate heavy metals (Saeid et al., 2012). It has been shown that cells within microalgae can be saturated with heavy metals within 24 hours (Kargi et al., 2006). However, there is not yet an official standard for the concentrations of heavy metals allowed within microalgae products. It has been estimated that out of the 100,000 species of microalgae, only 35,000 have been analyzed for their toxicological properties (Munir et al., 2013). There are certain strains of microalgae, especially cyanobacteria including *M. aeruginosa, Anabaena flos-aquae* and *Aphanizomenon flos-aquae* that can be toxic to both humans and animals. Occurrences of human or animal poisoning by these species have only been related to toxic blooms of wild microalgae, and there have not been any associated cases with microalgae cultivated for consumption (Becker et al., 2004).

1.3.3 Weaning stress on nursery pigs

Weaning is the process of transitioning a piglet from a liquid, milk diet from the sow to a solid diet, which on commercial farms occurs at 14 to 28 days of age. Throughout this transition, pigs are subjected to social and environmental stress, including separation from the sow, transportation, handling, mixing and establishing a hierarchy with new litter mates; and fluctuating temperatures. The significant change in diet and environment during weaning has been known to have drastic effects on feed intake, performance, digestive capacity and intestinal integrity.

Weaning stress is characterized by 100 to 250 g of body weight loss, which can lead to poor performance in subsequent stages of growth. Kats et al., (1992) reported that piglets gaining adequate weight at 225-340 g/d during the first week post weaning subsequently reached market weight 10 to 28 days earlier than piglets gaining poorly (zero to 110 g/d) during their first week after weaning. During these periods of low feed intake, metabolizable energy (ME) intake is not enough to meet the piglet's requirement to maintain or gain weight. It is estimated that at one-week post weaning, the ME intake of a piglet is about 60-70% of pre-weaning milk intake and can take up to two weeks to fully recover (Campbell et al., 2013).

Digestive capacity related to production of digestive enzymes and gastric acidity is affected by weaning. Montagne et al., (2007) showed that lactase activity in the piglets' small intestine decreased from 59 IU/mg protein at weaning to 5 IU/mg protein 15 days post weaning. In contrast, amylase activity increased from 20 to 43 IU/mg from days five to 15 post weaning. Similarly, the gastric pH in the stomach of piglets is highest at birth to digest colostrum, decreases to four from three to four

weeks post weaning, and is insufficient to digest protein (Suiryanrayna & Ramana, 2015; Mavromichalis, 2016).

Weaning also affects the structure and integrity of the intestine. Hampson, (1986) observed a 25-35% reduction in villus height in the intestine of weaned piglets compared to unweaned piglets. Pluske et al., (1996c) observed acute structural changes in the intestine in post-weaned pigs, characterized by shortening of the villi and increase in crypt depth induced by low voluntary feed intake. Gu et al., (2002) studied the effects of weaning age on small intestinal villus morphology. They observed a significant ($P< 0.01$) reduction in villus height in the duodenum, distal jejunum and ileum of pigs 29 days post weaning, and crypt depth increase until 50 days post weaning. An acute, degenerative phase can be observed as early as two days post weaning, and leads to a 20 to 30% loss in small intestinal mucosa weight (Lallès et al., 2004).

1.3.3.1 Nursery pig diet requirements

Swine producers typically utilize a three-phase feeding system for nursery pigs, which is based on weight of the pigs and feed budget. An example of a three-phase system based on body weight is as follows: 1.) 4-5 kg 2.) 5-7 kg 3.) 7-11 kg. These phases allow for a gradual transition from liquid to complex diets that match the digestive capacity of nursery pigs. Phase feeding of complex diets compared to feeding a simple corn-soybean meal diet can lead to an additional weight increase of one to two kg in pigs exiting the nursery stage (Coffey et al, 2000).

Nursery pigs have a high capacity for protein deposition compared to the level of feed intake, and have amino acid requirements, rather than crude protein requirements

(Derouchey et al., 2010). Most cereal grains fed in nursery pig diets are deficient in amino acids such as lysine, threonine and tryptophan. As lysine is the first limiting amino acid, diets are formulated to the ratio of methionine, cysteine, threonine, tryptophan, isoleucine, valine and histidine to levels of lysine. Phase one nursery pigs diets are recommended to provide 1.3 to 1.4% standardized ileal digestible (SID) lysine, equating to 20 to 22% crude protein (Coffey et al., 2000; Nemechek et al., 2011). Soybean meal is commonly added to phase one nursery pig diets as it provides high levels of crude protein and lysine at less cost, at 48% and 3% inclusion on an as fed basis, respectively (Cervantes-Pahm and Stein, 2010). Specialty protein sources are added to provide balance of amino acids including soy protein concentrate, fish meal, spray-dried plasma in addition to synthetic amino acids used to reduce crude protein levels and feed cost in nursery pig diets without negatively affecting growth performance (Frantz et al., 1968; Stoner et al., 1990; Lordelo et al., 2008; Waguespack et al., 2012).

Feeding highly digestible and palatable sources of energy is required to stimulate feed intake, as weaned pigs do not consume enough feed to meet their energy requirements (DeRouchey et al., 2010). The National Research Council (NRC,2012) recommendations for energy in phase one nursery pig diets is 3,400 kcal/kg of ME. Feeding fats and oils is a common strategy to increase the energy density of diets, as fats have approximately 2.25 times the amount of ME per unit of weight than carbohydrates from cereal grains (Reese et al., 2010). Common sources of lipids include those derived from animal fats such as poultry fat, tallow, choice white grease or oils from plants such as soybean, canola and corn oils. Although high in energy,

apparent total tract digestibility of animal fats increases with age more so than vegetable fats. In addition, peroxidation of lipids can cause decreases in growth performance in nursery pigs (Cera et al., 1988; Chang et al., 2016). Therefore, to prevent deleterious effects, recommended levels of lipids added to the diet to increase caloric intake in nursery pigs range from 3% to 5%.

Carbohydrates are a primary source of energy in nursery pig diets. Levels of digestive enzymes in nursery pigs vary throughout weaning, which directly impacts the carbohydrate sources they can utilize. Crystalline lactose or sources of lactose such as dried whey are fed in the early nursery phase as highly digestible and palatable sources of energy to increase feed intake. Phase one of nursery pig diets normally contain approximately 18-20% lactose, which has been proven to improve growth performance in weaned pigs (Tokach et al., 1995; Mahan et al., 2004). A large proportion of the carbohydrates in corn are starch, at approximately 680 g/kg of DM (Navarro et al., 2019). Therefore, corn should be limited in phase one, due to low amylase activity for proper digestion in weaned piglets.

Macrominerals are supplemented in larger quantities in nursery pig diets, and include calcium, phosphorus, sodium, chlorine, magnesium and potassium. Microminerals, otherwise known as trace minerals include zinc, copper, iron, manganese, iodine and selenium (Reese et al., 2010). Due to concerns of variability and bioavailability of minerals from feed ingredients such as corn and soybean meal, diets are typically formulated above the NRC (2012) recommendations to provide a safety margin (Flohr et al., 2016). Calcium and phosphorus are two of the most important macrominerals

which levels should be monitored in nursery pig diets, as a deficiency of one can affect how the other is utilized (Crenshaw, 2001; Menegat et al., 2019).

Average daily feed intake of nursery pigs in phase one is assumed to be 150 g/d, increasing to 250 g/d and 500 g/d in phase two and three, respectively (Reese et al., 2010). Phase two and three represent approximately 50% of total feed cost in nursery pig diets. During these phases, the goal in addition to providing a balanced diet, is to reduce feed cost by decreasing the levels of specialty feed ingredients and overall diet complexity.

In addition to nutrient levels, there are two primary factors to take into consideration when investigating the quality of nursery pig feed ingredients. Those are the presence of antinutritional factors and diet flowability. Anti-nutritional factors are substances in feed that can interfere with nutrient digestibility and utilization. Examples of anti-nutritional factors include non-starch polysaccharides (NSP), phytate, and trypsin inhibitors (Boggess et al., 2018). Flowability is the "relative movement of a bulk of particles among neighboring particles or along the container wall surface" (Peleg, 1977). Flowability of a feed ingredient is measured by the angle of repose, which is defined as the angle of the free surface of a heap of particulate material to the horizontal plane (McGlinchey, 2005). The characteristics of feed ingredients that most affect flowability are moisture content and particle size. A decrease in flowability reduces availability of feed in feeders, potentially decreasing feed intake in nursery pigs.

1.3.3.2 Microalgae as an alternative feed ingredient

The nutritional composition of microalgae is of particular interest to swine producers, as protein, carbohydrate, lipids and micronutrient levels in microalgae are comparable and, in some instances, greater than corn and soybean meal (Table 1.2). Challenges throughout weaning related to low feed intake, digestive stress, and necessity of highly digestible and palatable ingredients could be alleviated by incorporation of high-quality nutrients from microalgae in swine production systems. Positive effects of incorporating microalgae in swine production systems are described in section 1.4.4. An area of concern with respect to the nutritional composition of microalgae is that it is highly variable from species to species. Nutrient composition and availability in culture, temperature, light intensity, and CO_2 levels all can affect the nutritional composition of microalgae, especially lipid and carbohydrate contents (Becker et al., 2004). An example of this variability can be seen in table 1.2, which varying levels of nutrients between common species of microalgae.

a.) Protein and amino acids

The level of crude protein in microalgae can range from 10-70% on a DM basis (Becker et al., 2004). Comparatively, soybean meal typically has a maximum amount of crude protein of 48% on a DM basis. *In vitro* protein digestibility of common microalgae species such as *Arthrospira* and *Chlorella* has been analyzed, ranging from 70-85% (Tibbetts et al., 2016; Wild et al., 2018). Digestible protein from microalgae could potentially replace and therefore alleviate the harmful effects of soybean meal such as antinutritional factors and undigestible crude protein in early weanling pig diets. Microalgae also have the potential to synthesize nearly all amino acids (Spolaore

et al., 2006), with levels comparable to soybeans (Table 1.3), with the exception of methionine, and cysteine (Lum et al., 2013). Therefore, supplementation of synthetic amino acids or balance with complementary sources of protein in combination with microalgae is warranted, especially in early phase nursery pig diets.

b.) Lipids

Microalgae have elevated lipid levels compared with corn and soybean (Table 1.2), with a normal range of 1 - 40%, and a maximum 85% on DM basis (Metting et al., 1996). Lipids from microalgae are predominately grouped into two categories: storage lipids and structural lipids. Triglycerides (TAG) make up most of the storage lipids, which are composed of mainly saturated fatty acids and unsaturated fatty acids (Spolaore et al., 2006). These unsaturated fatty acids are a source of biodiesel, via transesterification (Chisti et al., 2007). Structural lipids, like phospholipids and sterols, are used and investigated in human and animal nutrition for their high level of polyunsaturated fatty acids (PUFAS) (Sharma et al., 2012). For example, high levels of linoleic acid, gamma-linolenic acid and eicosapentaenoic acid (EPA) can be found in many species of microalgae, which are of value to both human and animal health as they cannot be synthesized in the body (Table 1.5). There is however, a lack of research involving the *in vivo* digestibility of microalgae lipids. Skrede et al., (2011) stated that lipid digestibility decreased as the inclusion of three sources of microalgae increased in the diet of minks. This study provided insight into the resistance in digestibility of microalgae lipids due to their rigid cell walls.

c.) Carbohydrates

Levels of carbohydrates in microalgae range from 8 - 30% on a DM basis (Table 1.2), and can be found in the outer cell wall of microalgae (pectin, agar, alginate), the inner cell wall (cellulose, hemicellulose), and inside the cell (starch). The cell wall of microalgae also contains matrix polysaccharides, which include sulfated polysaccharides (Fernandez et al., 2017). Microalgae are studied as sources of digestible carbohydrates such as starch to provide energy from glucose and as indigestible sources, leading towards fermentation and available sources of energy from short chain fatty acids (SCFA) (Table 1.4). Levels of starch in *Chlorella vulgaris* have been observed as high as 55 % of total carbohydrates (Chen et al., 2013). Likewise, a recent analysis of the carbohydrate content from 16 species of microalgae showed ranges in total dietary fiber from 9 - 58% on a DM basis (Molino et al., 2018) (Table 1.4).

d.) Vitamins and minerals

Species of microalgae are either vitamin auxotrophs, meaning they require an exogenous source of vitamins in their environment for efficient growth, or protrophs, which can synthesize vitamins sufficiently (Tandon et al., 2017). Consequently, some microalgae are rich in a wide variety of vitamins such as vitamin A, B1, B2, B12, C, and E (Table 1.6). Fluctuations in vitamin content between different species have been attributed to the effects of processing of algae biomass such as drying, especially in relation to heat instable vitamins such as vitamin B1, B2, and C (Spruijt et al., 2016). Microalgae also provide essential minerals, such as calcium, phosphorus, and sodium (Table 1.7). It is known that many plant-based feed ingredients store phosphorus as

phytate, which is indigestible to monogastrics. Comparatively, microalgae have been researched as bioavailable sources of phosphorus, as they store inorganic phosphorus in vacuoles as polyphosphate granules, which is degradable by mammalian phosphatases (Morrissey et al., 2012; Tibbets et al., 2018). Therefore, microalgae have the potential to be a digestible source of phosphorus for swine.

1.4 Previous research in microalgae uses for humans and livestock

1.4.1 Microalgae use in humans

It is estimated that 70% of microalgae biomass currently produced each year is used for human nutritional supplements in the form of powders, tablets, and capsules. Common species of microalgae that have been generally regarded as safe by the FDA are *Chlorella, Spirulina, Dunaliella, Haematococcus,* and *Schizochytrium* (Chacón-Lee et al., 2010).

Aside from its uses as a protein supplement, *Spirulina* also has high levels of γ-linolenic acid and antioxidants, proven to lower the overall glycemic and lipidemic indices in human diets (Parikh et al., 2001). *Chlorella* is another species used as a protein supplement and as immunostimulator. In a randomized double-blinded placebo trial, patients (n = 23) were either provided five g of *Chlorella* or a placebo (n = 28) for a total of eight weeks. *Chlorella* supplementation increased ($P < 0.05$) the activity of natural killer cells by approximately 10% compared with placebo patients (Kwak et al., 2012). *Chlorella* has also been shown to reduce blood cholesterol levels and have beneficial antioxidant effects in smokers (Wells et al., 2017).

1.4.2 Poultry

Microalgae are utilized in poultry diets primarily as protein supplement, as research has shown that *Chlorella* can be used to partially replace commonly used protein sources at up to 10% of poultry rations (Spolaore et al., 2006). In recent years, the use of defatted microalgae in poultry diets has been investigated. Austic et al., (2013) studied the effects of a defatted diatom microalgal (DFA) biomass when replacing soybean meal in broiler chick diets. Two day old chicks were fed a control diet, 7.5% DFA to replace soybean meal, or 7.5 and 10% DFA to replace soybean meal and corn. Results showed that chicks fed diets containing DFA had significantly ($P< 0.05$) lesser body weight gain than those fed the control diet. Decreases in growth performance in the starter period for broiler chicks fed the DFA was thought to be caused by deficiencies in amino acids such as lysine and methionine, which is common in defatted microalgae sources. A follow up study was performed to investigate the effects of replacing soybean meal with 7.5% DFA, when adding 0.05% higher levels of synthetic amino acids such as DL-methionine and L-lysine (Austic et al. 2013). It was then observed that 7.5% DFA with higher supplemented amino acids had similar growth performance to those fed the control diets. Other uses of microalgae in poultry diets focus on specific compounds in microalgae, for example omega-3 fatty acids. Laying hens are fed microalgae sources rich in omega-3 fatty acids, which in turn lead to an egg that is rich in cholesterol-lowering omega 3 fatty acids (Spruijt et al., 2016).

1.4.3 Dairy and beef

The use of microalgae in dairy cow and steer diets has been also investigated. *Spirulina* has been utilized in dairy cows for its beneficial effects on growth performance and milk productivity. Kulpys et al., (2009) compared dairy cows fed either a control diet or a control diet with 200 g of *Spirulina* added daily and observed an overall increase in body weight gain by 8.5 - 11% and a 21% increase in milk yield compared with cows fed a control diet.

Microalgae are also used in dairy cow diets to increase the levels of polyunsaturated fatty acids, such as docosahexaenoic acid (DHA) in milk (Lum et al., 2013). By supplementing 105 g of *Aurantiochytrium limacinum*/cow/d, Moran et al., (2018) were successful in enriching milk in dairy cows with 4.7 mg of DHA/100 g of milk. Comparatively, average DHA content in milk from conventional dairy cows is estimated to be 0.6 mg DHA/100 g of milk (Benbrook et al., 2018).

Van Emon et al., (2015b) studied the effects of replacing corn with a de-oiled, heterotrophic algae meal (ALG) at 15%, 30% or 45% in steer diets. Over the 55-day experiment, no significant differences were found in midpoint and final body weight between control and any inclusion of the ALG. There was however a significant (P <0.001) increase in dry matter intake from 7.19 kg/d in the control group to 8.85 kg/d with 45% inclusion of ALG. It was concluded that the ALG was a digestible and viable feedstuff for steers.

1.4.4 Pigs

The main focus of feeding microalgae to pigs has been as a protein replacement in diets. Lee et al., (1979) fed pigs with *Micractinium* and *Scenedesmus,* microalgae that have elevated protein levels of approximately 60% on a DM basis. The results showed that pigs fed 8 % of each algae had growth performance that was comparable to the control diet of corn and soybean meal. However, 15% inclusion level of the microalgae resulted in a decrease in overall weight gain. Yap et al., (1982) fed a mixture of *Spirulina maxima* and *Anthrospira platensis,* or *Chlorella* to weaned pigs, with a goal of replacing 33% of soy protein in the basal diet. Data of overall growth performance, incidence of diarrhea, and toxicity levels were recorded. There were no significant differences in weight gain between weaned pigs fed the basal diet or microalgae diets. Furthermore, no cases of diarrhea or toxicity were observed. The authors concluded that the microalgae meal could replace up to 33% of the protein supplied by soybean meal in the diet, without causing detrimental effects to the pigs' health.

Microalgae are also researched as potential prebiotics in nursery pig diets. Furbeyre et al., (2016) investigated the effects of adding microalgae, specifically 1% *Spirulina* or 1% *Chlorella* on growth performance and intestinal development in weaned piglets. Inclusion of *Spirulina* or *Chlorella* did not improve growth performance. However, incidence of diarrhea was reduced ($P<0.01$) from 36% in the control group to 24% in pigs fed 1% *Chlorella.* The villus height in the jejunum ($P<0.05$) increased from 481 µm in control pigs to 524 µm and 523 µm in pigs fed *Spirulina* and *Chlorella,* respectively. In addition, the villus height to crypt depth ratio in the jejunum ($P<0.05$)

increased from 1.59 in the control group to 1.93 and 1.85 in the *Spirulina* and *Chlorella* groups. This study provided insight into the potential use of microalgae as a tool to relieve digestive damage caused throughout weaning (Furbeyre et al., 2016). The use of microalgae byproducts from the biofuel industry is a relatively new area of research in the swine industry. Isaacs et al., (2011) investigated the use of 7.2% defatted and 6.6% full fat biomass from *Staurosira* spp. to replace soybean meal in 27 weanling pig diets. No significant differences were observed in body weight gain or health status in the weanling pigs over the six week study, leading to the conclusion that either source could safely be used to replace soybean meal in the diet. Similarly, Lum et al., (2012) fed a DFA, in order to determine the maximum inclusion level in weanling pig diets. Weanling pigs were fed 7.5% DFA or 15% DFA, replacing corn and soybean meal, for a total of six weeks. Weanling pigs that were fed 15% DFA showed an 11% reduction in ADG and G:F, while pigs fed 7.5% DFA had a 9% reduction in ADG and G:F, with no overall difference in ADFI. Further studies using microalgae byproducts from the biofuel industry are needed, in order to define the maximum inclusion level and to investigate the potential negative impacts involved with supplementation in swine diets.

The fatty acid profile of microalgae has also been researched to improve pork meat quality characteristics. Sardi et al., (2006) evaluated the effects of a marine algae product (MA) containing high levels of DHA on meat and subcutaneous fat quality in heavy pigs (118 ± 6.7 kg). Sixty pigs were fed four diets: a control diet (corn/soybean meal), 2.5 g of algae/kg of the diet eight weeks prior to slaughter, or five and 2.5 g of algae/kg of the diet over the last four weeks prior to slaughter. Results showed that

inclusion of the marine algae product (MA) did not affect growth or meat characteristics of the pigs (pH value, meat color, loin composition, or iodine number in subcutaneous fat). However, pigs fed the MA showed DHA levels ranging from 40 to 70 mg/100 g in *longissumus dorsi* and 80 to 130 mg/100 g in subcutaneous fat. The researchers concluded that the inclusion of the MA in swine diets could provide added levels of DHA in pork, which according to the USDA typically only contains 11.3 g of fat/100 g of meat, with 1.3 g of polyunsaturated fats (Meadus et al., 2013).

Overall, microalgae can provide high quality nutrients for pigs, and benefits related to growth performance, health status and meat characteristics. However, additional information is needed, especially *in vivo* digestibility of carbohydrates and lipids, to determine the overall potential of microalgae to supply energy in swine diets. Further classification of carbohydrates from microalgae is also needed to truly understand their potential biological effects.

1.4.5 Aquaculture and companion animals

Additional uses of microalgae are seen in the aquaculture market and companion animal feed industry. Species of microalgae such as *Schizochytrium sp.* have been used as a fortifying agent in dog diets to improve cognitive function and eye sight (Hadley et al., 2017). Microalgae are also added to pet food diets to enhance external characteristics of dogs and cats, such as skin and coat health (Gouveia et al., 2008). The aquaculture market uses microalgae as a feed source and as a specialty ingredient for their pigment content. Species of microalgae such as *Pavlova* sp. and *Isochrysis* sp. are the most common sources of food for marine animals during their life cycle

(Priyadarshani et al., 2012). *Haematococcos pluvialis* is utilized in fish diets for its astaxanthin content (Van Iersel et al., 2010), which gives species of fish such as wild salmon and trout the typical red muscle color that fish markets desire. Cultivation systems that co-produce microalgae and fish are being researched, thereby producing a system with limited waste and access to bioenergy (Van Iersel et al., 2010).

1.5 Summary

Microalgae meet the criteria set for alternative feed sources. Their protein, carbohydrate, and fat content can be seen at higher levels than corn or soybean, which provide added benefits in terms of possible growth performance for animal production systems. In addition, health promoting compounds within microalgae, such as polysaccharides and fatty acids show promising benefits for use in animal diets, especially in swine diets as a possible alternative as growth promoters. They also have the potential to significantly reduce the environmental impact that animal production systems have, due to their ease of production and the decrease in resources needed for their production, such as water and arable land. Further research is needed to determine the optimal source and inclusion level of microalgae for animal diets. With a focus on investigating the potential advantages of microalgae in nursery pig diets, this experiment utilized a microalgae extract (MAE) obtained after partially de-oiling microalgae for biofuel production. The aim of this study was to understand how the MAE can be used either as a feed ingredient or as a health promoter in nursery pig diets, with the objective to determine the optimal inclusion of the MAE.

Table 1.1 Classification of commercially used microalgae

Kingdom	Class	Species used commercially
Chromista	*Bacillariophyceae*	*Phaedactylum tricornutum*
Plantae	*Chlorophyceae*	*Chlorella vulgaris*
Chromista	*Eustigmatophyceae*	*Nannochloropsis oculata*
Chromista	*Haptophyceae*	*Isochrysis galbana*
Chromista	*Chrysophyceae*	*Cryptomonas rufescens*
Bacteria	*Cyanophyceae*	*Spirulina maxima*
Protozoa	*Euglenophyceae*	*Euglena gracilis*

Adapted from Heimann and Huerlimann, (2015)

Table 1.2 Comparisons of crude protein, carbohydrate, and lipid composition from various sources of microalgae with corn and soybean (% dry matter)

Item	Source-Processing Method	Crude Protein	Carbohydrates	Lipids	Reference
Soybean	-	37	30	20	Lum et al., 2013
Corn	-	10	85	4	Lum et al., 2013
Anaebaena cylindrical	Freshwater biomass-dried	43-56	25-30	4-7	Lum et al., 2013
Spirulina maxima	Open pond cultivation	60-71	13-16	6-7	Lum et al., 2013
Chlorella vulgaris	Waste water collection	51-58	12-17	14-22	Lum et al., 2013 Van Iersel et al., 2010
Staurosira sp.	De-fatted biomass, biofuel co-product	19	14-15	3-4	Austic et al., 2013
Crypthecodinium sp.	Glucose/acetic acid cultured, dried, oil extraction	12-15	40	40-50	Van Iersel et al., 2010 Pleissner et al., 2012

Table 1.3 Amino acid composition of different species of microalgae (g/100 protein) and soybean

AA	Soybean	*Dunaliella bardawil*	*Chlorella vulgaris*	*Spirulina platensis*
Ala	5.0	7.3	9.4	9.5
Arg	7.4	7.3	6.9	7.3
Asp	1.3	10.4	9.3	11.8
Cys	1.9	1.2	-	0.9
Glu	19.0	12.7	13.7	10.3
Gly	4.5	5.5	6.3	5.7
His	2.6	1.8	2.0	2.2
Ile	5.3	4.2	3.2	6.7
Leu	7.7	11.0	9.5	9.8
Lys	6.4	7.0	6.4	4.8
Met	1.3	2.3	1.3	2.5
Phe	5.0	5.8	5.5	5.3
Pro	5.3	3.3	5.0	4.2
Ser	5.8	4.6	5.8	5.1
Thr	4.0	5.4	5.3	6.2
Trp	1.4	0.7	-	0.3
Tyr	3.7	3.7	2.8	5.3
Val	5.3	5.8	7.0	7.1

Adapted from Lum et al., (2013) and Becker, (2004)

Table 1.4 Total dietary fiber, starch, and monosaccharide composition of various species of microalgae

Microalgae	TDF (% DM)	Starch (% DM)	Gal	Glc	Man	Ara	Rib	Xyl	Fuc	Rha	Ref
Chlorella vulgaris Strain A9	-	-	4.39	19.92	< Q	ND	0.57	0.72	ND	1.39	Schulze et al., 2017
Dunaliella salina	-	-	2.18	21.06	ND	ND	0.75	0.76	ND	ND	Schulze et al., 2017
Scenedesmus quadricauda	-	-	1.99	11.91	8.92	ND	ND	ND	ND	ND	Schulze et al., 2017
Chlorella vulgaris Strain UTEX 395	-	-	7.1 ± 0.1	5.1 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	ND	0.8	ND	ND	Templeton et al., 2012
Nannochloropsis sp.	-	-	1.8	3.9 ± 0.1	0.2	ND	ND	ND	ND	ND	Templeton et al., 2012
Phaeodactylum tricornutum	-	-	1.9 ± 0.2	2.6± 0.2	8.6± 0.4	1.1± 0.1	ND	1.8	ND	ND	Templeton et al., 2012
Chlorella vulgaris	35.04 ± 1.60	-	-	-	-	-	-	-	-	-	Molino et al., 2018
Spirulina platensis	42.82 ± 1.20	-	-	-	-	-	-	-	-	-	Molino et al., 2018
Dunaliella salina	8.97 ± 0.50	-	-	-	-	-	-	-	-	-	Molino et al., 2018
Chlamydomonas reinhardtii	-	45.0	-	-	-	-	-	-	-	-	Hirano et al., 1997
Chlorella vulgaris Strain IAM C-534	-	37.0	-	-	-	-	-	-	-	-	Hirano et al., 1997
Scenedesmus sp.	-	20.4	-	-	-	-	-	-	-	-	Rodjaroen et al., 2007

Monosaccharides are mean ± standard deviation for triplicate hydrolyzes of 100 mg of dry biomass. ND: Not detected, -: not reported, < Q: Detected, below the quantitation limit

Table 1.5 Fatty acid content of different species of microalgae (% of fatty acids from oil extraction)

Fatty acid	*Dunaliela bardawil*	*Chlorella vulgaris*	*Spirulina platensis*
Lauric acid 12:0	-	-	0.04
Myristic acid 14:0	-	0.9	0.7
Pentadecanoic acid 15:0	-	1.6	trace
Palmitic acid 16:0	41.7	20.4	45.5
Palmitoleic acid 16:1	7.3	5.8	9.6
Hexadecatetraenic acid 16:4	3.7	-	-
Heptadecanoic acid 17:0	2.9	15.3	0.3
Stearic acid 18:0	2.9	15.3	1.3
Oleic acid 18:1	8.8	6.6	3.8
Linoleic acid 18:2	15.1	1.5	14.5
α-Linolenic acid 18:3	20.5	-	0.3
γ-Linolenic acid 18:3	-	-	21.1
Eicosapentaenoic acid 20:5	-	20.8	0.4

Adapted from Lum et al., (2013) and Becker, (2004)

Table 1.6 Vitamin content from different species of microalgae (mg/kg dry matter)

Vitamin	*Chlorella pyrenoidosa*	*Scenedesmus quadricauda*	*Spirulina platensis*
Vit A	480	554	840.0
Vit B1	10	11.5	44.0
Vit B2	36	27	37.0
Vit B6	23.0	-	3.0
Vit B12	-	1.1	7.0
Vit C	-	396	80
Vit E	-	-	120
Biotin	0.15	-	0.3
Folic acid	-	-	0.4
Nicotinate	240	108	-
Pantothenic acid	20	46	13.0

Adapted from Lum et al., (2013) and Becker, (2004)

Table 1.7 Mineral content of different species of microalgae

Mineral	Soybean meal	*Spirulina maxima*	*Chlorella vulgaris*	*Haematococcus pluvialis*
Ca[1]	0.31	0.91	4.73	0.25
K[1]	2.05	2.58	0.98	0.97
Mg[1]	0.28	0.35	1.46	0.22
Na[2]	127	8.53	0.98	5.87
P[1]	0.67	1.29	1.53	1.31
Cu[2]	15	1.1	2.2	344
Fe[2]	172	93.6	166.3	822.7
Mn[2]	41	24.6	471.5	111.9
Zn[2]	48	3.5	17.5	232.2

[1] % as fed [2] mg/kg as fed-basis. Adapted from Batal et al.,(2010) and Spruijt et al., 2016

Chapter 2. Evaluation of a partially de-oiled microalgae product in nursery pig diets

2.1 Abstract

Although microalgae can be used as a source of energy and macronutrients in pig diets, there is limited information on the use of partially de-oiled microalgae co-products in swine feeding programs. The objectives of this study were to evaluate the effects of a partially de-oiled microalgae extract (MAE) in nursery pig diets on growth performance and health status. A total of 300 pigs (initial BW = 6.3 ± 2.1 kg) were used in a 42-d experiment. Treatments included a standard corn-soybean meal control diet, and diets containing 1, 5, 10, or 20% MAE replacing primarily corn. The ME content of MAE was calculated from the chemical composition, and diets were formulated to meet or exceed nutrient requirements for nursery pigs. Pigs were stratified by weaning BW into 12 blocks in a randomized complete block design, with sex distributed evenly among blocks. Pens of pigs (5 pigs/pen) were assigned randomly within block to one of 5 dietary treatments. Pig BW and feed disappearance were recorded weekly. On d 42, thirty pigs were harvested and sections of the jejunum and ileum were collected for gut morphology analysis, and a liver sample was collected for metabolomic analysis using liquid chromatography-mass spectroscopy. Data were analyzed by ANOVA with diet as treatment effect, and contrasts were used to test linear or quadratic effects of dietary MAE inclusion level. Overall, pigs fed 1 and 10% MAE had the greatest (quadratic $P < 0.05$) ADG, resulting from greater (quadratic $P < 0.05$) ADFI. There was a tendency for a greater number of pigs requiring injectable treatments ($P = 0.16$) and a greater mortality ($P = 0.14$) in pigs fed the control diet than pigs in any of the diets with the MAE. Final

BW increased ($P < 0.05$) for pigs fed 1 and 5% MAE diets. The improvements in ADG were not explained by differences in mucosa height or goblet cell count among dietary treatments. Pigs fed diets containing 1 or 5% MAE had relatively less concentration ($P < 0.05$) of ammonia in the liver and had changes in metabolites associated with the urea cycle. In conclusion, feeding MAE resulted in increased growth responses and may have beneficial health effects when fed to nursery pigs.

2.2 Introduction

Microalgae are single celled microorganisms with multiple industrial uses, such as biofuel production (Gatrell et al., 2014) and wastewater remediation (Lu et al., 2015;Lu et al., 2016). Likewise, interest in using microalgae co-products in animal feeding programs is increasing because microalgae grow rapidly and can sequester and convert carbon dioxide into energy and nutrient rich biomass that can be later fed to livestock at lower carbon, water, or land footprint than common feed ingredients (Gatrell et al., 2014). Depending on the specific species, cell fraction, and processing methods used, microalgae contain variable concentrations of protein (10 -70%), lipids (1 – 40%), and carbohydrates (8 – 30%), as well as vitamins and trace minerals in forms that appear to be highly bioavailable (Becker, 2004). Microalgae and their co-products have also been shown to have several functional properties that include serving as antibacterial, antiviral, and antioxidant agents (Ma et al., 2015; de Jesus Raposo et al., 2016). Therefore, microalgae and their co-products could be used not only as sources of energy and nutrients, but also as sources of nutraceuticals and prebiotic carbohydrates in animal feeds (Yang et al., 2014).

Unfortunately, there is limited information on the benefits and limitations of various feeding applications of microalgae co-products in swine diets (Gatrell et al., 2014). Early studies showed that feeding some species of microalgae to pigs had no negative effects on growth performance (Hintz and Heitman, 1967; Fevrier and Seve, 1975) and replacing soy protein in weaned pig diets with a microalgae mixture resulted in no diarrhea or gastrointestinal lesions (Yap et al., 1982). More recent studies have shown that feeding either full-fat or defatted microalgae biomass to weaned pigs either had no effect on growth rate and health status (Isaacs et al., 2011) or reduced growth performance (Lum et al., 2013). The lack of adequate nutrient profile (content and digestibility) may explain the decrease in growth performance when new feed ingredients are evaluated. Likewise, microalgae biomass could have nutritional beneficial properties that are unknown. Therefore, the aim of this study was to evaluate the potential use of microalgae extract (MAE) as a feed ingredient in nursery pig diets.

2.3 Materials and methods

All experimental procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee (Protocol No. 1411-32072A).

2.3.1 Ingredient

The MAE co-product used in this study was provided by Solazyme Inc. (TerraVia, San Francisco, CA). The MAE product was analyzed using standard procedures (AOAC, 2012) for moisture (Method 030.15), CP (Method 990.03), ether extract (EE; Method 920.39), and ash (Method 942.05) content, complete amino acid profile (Method 982.3; including sections a, b, and c for hydrolysis of cysteine, methionine, and tryptophan), and

complete fatty acid profile (Method 996.06; Table 2.1) by the New Jersey Feed Laboratory, Inc. (Ewin Township, NJ).

2.3.2 Glycosyl composition

Carbohydrate composition of MAE was analyzed using several different assays (Table 2.1). Briefly, the glycosyl composition of the sample (1.5 mg) was analyzed before and after hydrolysis in 2 M trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO) at the Complex Carbohydrate Research Center (Athens, GA). The sample (1.5 mg) was placed in a screw-cap tube with 80 µg of inositol as internal standard and hydrolyzed in 2 M trifluoroacetic for 2 h in sealed tubes at 121 °C, reduced with sodium borodeuteride ($NaBD_4$), and acetylated using acetic anhydride/trifluoroacetic acid. A different sample (3.0 mg) was mixed with 160 µg of inositol as internal standard, directly reduced with $NaBD_4$, and acetylated using acetic anhydride/trifluoroacetic acid. The resulting alditol acetates were analyzed on an Agilent 7890A GC (Minnetonka, MN) interfaced to a 5975C MSD (triple-axis) in electron impact ionization mode. Separation was performed on a 30 m Supelco 2330 bonded phase fused silica capillary column (Sigma-Aldrich). The concentration of carbohydrates presumed to be indigestible in the small intestine, but degradable during fermentation in the hindgut, were analyzed by the total dietary fiber assay following procedure 985.29 (AOAC, 2012) and procedure 993.19 for the analysis of insoluble fiber. Soluble dietary fiber was calculated as the difference between total dietary fiber and insoluble dietary fiber.

2.3.3 Dietary treatments and experimental design

Dietary treatments included: 1) corn and soybean meal (**CON**), 2) CON with 1% MAE, 3) CON with 5% MAE, 4) CON with 10% MAE, and 5) CON with 20% MAE. Diets

were formulated to meet the nutrient requirements of nursery pigs and fed using a 3-phase program, where each phase consisted of a two-week period (Tables 2.2, 2.3, and 2.4). Diets for all phases were formulated with the MAE to partially replace corn and soybean meal. We adjusted diet ME by adding soybean oil to meet or exceed nutrient requirement recommendations for nursery pigs fed diets containing 3,400 kcal/kg of ME (NRC, 2012). The ME of MAE was estimated using a prediction equation based on chemical composition (Noblet and Perez, 1993):

$$ME\ (kcal/kg\ as\text{-}fed) = 4{,}194 - (9.2 \times ash) + (1.0 \times CP) + (4.1 \times EE) - (3.5 \times NDF)$$

where CP, EE, and NDF composition data (g/kg) were used on an as-fed basis.

Samples of complete diets were obtained after mixing, frozen at -20 °C, and analyzed for nutrient composition at the Experiment Station Chemical Laboratories (University of Missouri, Columbia, MO) for DM, CP, EE, crude fiber, and ash following AOAC procedures (AOAC, 2012). Concentration of Ca and P were analyzed using inductively coupled plasma – optical emission spectroscopy (Method 985.01; AOAC, 2012). The concentration of amino acids (including Trp, Met and Cys) were analyzed after appropriate hydrolysis [Method 982.30 including sections E (a, b, and c) in AOAC (2012)].

Diet flowability was determined by measuring the poured angle of repose (McGlinchey, 2005; Jiang and Rosentrater, 2015). Briefly, at the time of diet formulation, a sample of each diet (1 kg) was collected in an air tight plastic bag and stored at -20 °C until analyzed. A modified Hele-Shaw cell was used to measure the angle between the horizontal base and the slope of the pile of feed that dropped from a height of 60 cm using a funnel, and the poured and drained angles of repose were calculated (Johnston et

al., 2009). Measures of each diet were determined in triplicate and data are presented as means ± SD.

2.3.4 Animals, housing and management

Weaned pigs (n = 300; 21 days of age; 6.27 ± 0.02 kg) were selected from a batch of 400 pigs and blocked by initial body weight and allotted to 60 pens, with 5 pigs per pen in 12 blocks. Ratio of gilts and barrows was balanced evenly within blocks and treatments. Pens within blocks were assigned randomly to 1 of 5 dietary treatments. Pigs were housed in a temperature-controlled nursery facility at the University of Minnesota's West Central Research and Outreach Center in Morris, MN and were provided *ad libitum* access to feed and water throughout the entire 42-day experiment. Each pen (2.4 x 1.2 m) included plastic grated flooring, 1 cup drinker, and one 4-hole stainless steel self-feeder (Hog Slat Inc. Newton Grove, NC). Individual pigs in each pen were weighed once weekly to calculate pen average daily gain (ADG). All feed additions to feeders were weighed and remaining feed in the feeders was weighed the same day pigs were weighed and subtracted from feed added to determine feed disappearance and calculate average daily feed intake (ADFI) and gain to feed (G:F). Pigs were monitored daily for signs of poor health, and appropriate medication treatments were administered as prescribed by an attending veterinarian as needed.

2.3.5 Growth performance data collection and statistical analysis

Growth performance data were analyzed using the Mixed Procedure of SAS (v9.3; SAS Inst. Inc., Cary, NC), based on a randomized complete block design. Pen served as the experimental unit that was nested within block and diet, and was used as the subject for repeated measures with autoregressive covariance structure. Growth performance data

were analyzed using block as the random effect and treatment, week, and week × treatment interaction as fixed effects. Linear and quadratic contrasts were estimated using coefficients that were adjusted for the separation among dietary treatments. Iterations of models were tested modifying covariance structure and interactions using Bayesian Information Criterion to select the final model. The univariate test in SAS was used to evaluate the normality of residuals within the model, and to test for outliers and equal distribution in variance. Data are presented as the least squares means of each treatment in each week, and means were separated using the Tukey test adjusted for multiple comparisons.

Mortality and health treatment data were collected to assess health status by recording the number of pigs within each treatment group that received individual antibiotic. Treated pigs and pig mortality were calculated using the number of pigs that received individual treatments (0 vs. 1) or died (0 vs. 1), within each treatment, divided by the total number of pigs assigned to each dietary treatment (n = 60 pigs/treatment). Differences in treatment and mortality curves were tested using the Mantel-Cox Log-rank test in GraphPad Prism 7.03 (GraphPad Software, Inc., La Jolla, CA).

2.3.6 Tissue collection and analysis

Thirty pigs (6 pigs/treatment) were euthanized on d 42 by captive bolt followed by exsanguination; within a pen, the pig with the body weight closer to the pen average that did not received additional antibiotics was selected. Samples of the jejunum (1 m distal to the pyloric sphincter) and ileum (15 cm proximal to the ileocecal valve) were collected and fixed in 4% buffered formalin for histological evaluation. Liver samples (500 mg)

were collected from the left lateral lobe, snap frozen in liquid nitrogen, and stored at -80^{0}C for further processing.

Formalin-fixed intestinal samples were processed and embedded in paraffin following the standard protocols of the University of Minnesota's Comparative Pathology Shared Resource (St. Paul, MN). Tissue blocks were sectioned at 5 µm thickness and stained with hematoxylin and eosin (HE). Total mucosal height was measured from the tip of the villi to the bottom of the crypt on the HE stained sections at 100 X amplification under a light microscope (Olympus BX53). Data reported are the average of measurements of five well-oriented fields (fields that allow observing villi in their axis) per pig.

Five µm tissue sections were stained with periodic acid-Schiff with Alcian blue (PAS-AB, Newcomer Supply, Middleton, WI) following manufacturer's instructions. The stained slides were analyzed at 100 × magnification under a light microscope (Olympus BX53) in five well-oriented fields. Within each field, the total area (µm^2) of the mucosa was first measured, then the area (µm^2) stained positive for PAS-AB (goblet cell area) was determined by color using a cell imaging software (CellSense, Olympus, Center Valley, NJ). Mucosal area was defined as the area limited by the epithelial apical membrane and the *muscularis mucosa*. Data presented are the mean of the percentage of goblet cells in their corresponding mucosal area quantified in five fields per pig. Values for total mucosal length and goblet cell quantifications were analyzed using GraphPad Prism 7.03 (GraphPad Software, Inc., La Jolla, CA). Data were tested for normality using the D'Agostino and Pearson tests, and differences among groups were determined using the Kruskal-Wallis test followed by Dunn's multiple comparisons. For gut morphometry, pig was considered the experimental unit.

2.3.7 Metabolomic analysis

Aqueous fractions of liver were prepared using the Bligh and Dyer method (Bligh and Dyer, 1959). Briefly, 100 mg of frozen liver sample were homogenized in a mixture of 0.5 ml methanol, 0.5 ml chloroform and 0.4 ml distilled water. After 10 min of centrifugation at 18,000 × *g*, the top aqueous fraction was harvested and stored at -80°C. The aqueous fraction was derived by dansyl chloride (DC) for detecting amine-containing metabolites, including amino acids. Briefly, 5 µL of sample was mixed with 100 µL of DC (3 mg/ml in acetone), five µL of 50 µM internal standard d5-tryptophan and 50 µL of 10 mM sodium bicarbonate. This mixture was incubated at 60 °C for 15 min and subsequently centrifuged at 18,000 x *g* for 10 min. The supernatant was transferred into an HPLC vial for liquid chromatography-mass spectrometry analysis. Five µL of DC-derivatized sample was injected into an Acquity Ultra-Performance Liquid Chromatography (UPLC) system (Waters Corporation, Milford, MA) and separated in a BEH C18 column (Waters). The mobile phase for DC-derivatized samples used a gradient ranging from water to 95% aqueous acetonitrile containing 0.1% formic acid over a 10-min run. The LC eluant was introduced into a Xevo-G2-S quadrupole time-of-flight mass spectrometer (QTOFMS, Waters) for accurate mass measurement and ion counting. Capillary voltage and cone voltage for electrospray ionization was maintained at 3 kV and 30 V for positive-mode detection. Source and desolvation temperatures were set at 120°C and 350°C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (600 L/h), and argon was used as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution with mass-to-charge ratio (*m/z*) of 50-1,000 and monitored by the intermittent

injection of the lock mass leucine enkephalin ($[M+H]^+$ = *m/z* 556.2771) in real time. Mass chromatograms and mass spectral data were acquired and processed by the MassLynx^{TM} software (Waters) in centroided format. Additional structural information was obtained by tandem MS (MSMS) fragmentation with collision energies ranging from 15 to 40 eV.

For the metabolomic analysis, the chromatographic and spectral data were deconvoluted using the MarkerLynx software (Waters). A multivariate data matrix containing information on sample identity, ion identity (retention time and *m/z*) and ion abundance was generated through centroiding, deisotoping, filtering, peak recognition, and integration. The intensity of each ion was calculated by normalizing the single ion counts *versus* the total ion counts in the whole chromatogram. The processed data matrix was exported into SIMCA-P+^{TM} software (Umetrics, Kinnelon, NJ), transformed by *Pareto* scaling, and then analyzed by principal component analysis. Major latent variables in the data matrix were determined as the principal components of a multivariate model, and the relationships among examined samples were described in the scores scatter plot. Metabolite markers of MAE were identified by analyzing ions contributing to sample separation in the model. The metabolite structures were elucidated by accurate mass measurement, elemental composition analysis, database search (Human Metabolome Database, http://www.hmdb.ca), MS/MS fragmentation, and the comparisons with authentic standards. Amino acids were quantified by calculating the ratio between the peak area of amino acids and the peak area of internal standard and fitting with a standard curve using QuanLynx^{TM} software (Waters).

For all analyses, significant differences were considered at $P \leq 0.05$, with trends at $0.05 < P \leq 0.20$.

2.4 Results

2.4.1 Animal health and mortality

During the second week of the experiment, a high incidence of coughing and scouring was observed in pigs across all dietary treatments. Consequently, all pigs were treated with neomycin (22 mg/kg BW) by water medication from days 15 to 21 of the experiment. In addition to the neomycin treatment, 18 pigs were treated individually with enrofloxacin because of prevailing coughing and gaunt appearance. Of the 18 treated pigs, 8, 2, 3, 2, and 4 pigs were assigned to the CON, 1%, 5%, 10%, and 20% MAE diets, respectively (Figure 1). Overall mortality was 2.67% in this study, with 3, 2, 3, 0, and 0 pigs that died in the CON, 1%, 5%, 10%, and 20% MAE treatments, respectively. Trends for a greater incidence of medication treatment ($P = 0.16$) and mortality ($P = 0.14$) were observed in pigs assigned to the CON diet than for pigs fed any of the diets containing MAE. The calculated livability index (proportion of the total number of pigs that survived and did not receive additional injection treatment, expressed as percentage) were 81, 7, 93.3, 90, 96.7, and 93.3% for CON, 1%, 5%, 10%, and 20% MAE treatments, respectively.

2.4.2 Growth performance

There were no differences in initial BW among dietary treatments (Table 2.5). As early as d7, there were differences in BW among pigs because feeding MAE elicited a quadratic ($P < 0.05$) increase in BW. The increase in BW of pigs consuming 1% MAE observed at

day 7 was sustained in subsequent weigh periods. The net result was that the final BW of pigs among pens consuming MAE was greatest when consuming 1, 5, or 10% MAE compared with those fed the CON diet, but feeding 20% was not different from the CON diet (quadratic effect $P < 0.05$). The greater final BW appeared to be the result of greater ADG from d 1 and 7, where pigs had the greatest ADG when consuming the 1 and 10% MAE diets compared with feeding the other dietary treatments. The ADG of pigs consuming 20% MAE was less than those fed the 1, 5, or 10% MAE diets, and was not different from those fed the CON diet (quadratic effect $P < 0.05$). This greater ADG was likely a result of greater ADFI, where on d 7, the ADFI of pigs consuming MAE diets increased with increasing levels of MAE up to 10%, but feeding the 20% MAE diet resulted in similar ADFI compared with feeding CON (quadratic effect $P < 0.05$). As a result of greater ADFI, there were no effects of feeding MAE on G:F during most weigh periods. However, there was a linear increase ($P < 0.05$) in G:F in pigs fed the MAE diets during day 15 to 21. Total pen BW of pigs consuming 10% MAE (138 kg) were heavier ($P < 0.05$) than pen BW of pigs consuming the control diet (126 kg), while there were no differences in pen BW among pigs consuming 1, 5, or 20% MAE diets.

2.4.3 Diets

Diets containing 10% and 20% MAE were difficult to mix using a vertical screw mixer. As a result, these diets were mixed using a paddle mixer. The angle of repose was used as a measure of diet flowability and increased (linear $P < 0.05$) with greater dietary inclusion rates of the MAE from 37.7 ± 2.3, 44.3 ± 0.6, 48.0 ± 5.2, 50.0 ± 5.0, and 51.3 ± 5.5° for 0, 1, 5, 10, and 20% MAE, respectively. Because of the poor flowability of

experimental diets, all feeders were checked twice daily to ensure pigs had uninterrupted access to experimental diets.

2.4.4 Gut morphology

Feeding diets with MAE did not result in changes in intestinal architecture measured by the height of the intestinal mucosal ($P = 0.99$) or presence of mucus producing cells (goblet cell area, $P = 0.22$) in the jejunum. In contrast, the ileum of pigs fed the 5% MAE diet tended ($P = 0.06$) to have reduced mucosal height compared with that of pigs fed 20% MAE diet. Goblet cell area of the ileum was not affected by dietary treatments ($P \geq 0.05$, Figure 2).

2.4.5 Liver metabolite analysis

A PCA model on the hepatic metabolites revealed a dose-dependent separation of CON and MAE dietary treatments (Fig. 3A). The analysis of the relative abundance of metabolites derivatized by dansylation suggests that ammonia concentrations had a relatively large impact on the separation among treatments. However, these were not quantitative changes that describe the actual concentration of metabolites (data not shown). Consequently, we measured the concentration of free amino acids in the liver to determine their relative contributions to differences among dietary treatments. Our results showed differences in the quantitative concentration of hepatic alanine, arginine, histidine, ornithine, aspartic acid, citruline, and proline (Table 2.6). However, these differences in amino acid concentrations did not follow a pattern or appear to be associated with dietary inclusion level of MAE.

2.5 Discussion

The production and use of microalgae has long been recognized as a means to reduce the carbon footprint in biofuels production (Lum et al., 2013) bioremediation of waste-water (Chung et al., 1978; Lu et al., 2015;Lu et al., 2016), and other industrial applications (Mercer and Armenta, 2011). In addition, there is increasing interest in producing and using microalgae and derived co-products in animal feeds because large amounts of biomass can be rapidly produced from low value substrates, and result in an environmentally sustainable, highly nutritious feed ingredient for animals. However, the nutritional benefits and applications of microalgae biomass and co-products depend on the specific species of microalgae and industrial processes used to produce the co-products. Few studies have been conducted to evaluate the use of microalgae co-products in swine diets (Lum et al., 2013). Furthermore, the MAE used in this study has not been previously evaluated for use in swine diets. Consequently, we cannot discuss and compare the results obtained in this study with reference to other studies, but will focus the discussion on the product composition and our observations on health and growth of nursery pigs.

The microalgae co-product evaluated in this study was produced using proprietary mechanical lipid extraction procedures, which resulted in concentration of the carbohydrate content to about 76.62% on an as-fed basis. The concentration of total dietary fiber and glucose in the hydrolyzed extract suggested that carbohydrates in this microalgae co-product source were mostly of soluble glucose polysaccharides, such as starch, with the potential to be digestible in the small intestine and provide energy from glucose (Chen et al., 2013). The MAE also had glucose polysaccharides that were

insoluble and potentially related to cellulose, which could be fermentable in the large intestine of pigs. Because microalgae has also been studied as a potential source of prebiotics (de Jesus Raposo et al., 2016), we added it to common nursery diets at low inclusion rates (1% and 5%) to determine if potential prebiotic effects may improve growth performance and health. We also added MAE at high (10% and 20%) inclusion levels to determine its feeding value as an energy source in nursery pig diets.

One of the major findings of this study was the improvement in ADFI when pigs were fed diets containing MAE at low inclusion (1 and 5%) rates. In this preliminary experiment, we did not measure specific mechanisms that might explain this feed intake response. However, we speculate that the quadratic ADG and ADFI may be related to the carbohydrate composition of the MAE, which may have improved palatability of the diet. The quadratic response of ADFI and ADG observed may be related to dietary starch content. While increasing the inclusion of MAE, the type of starch from corn is replaced by starch from MAE. The type of starch and degradation kinetics during small intestine digestion may be different and impact feed intake and subsequent growth performance of nursery pigs (Zijlstra et al., 2012; Fouhse et al., 2017). More investigation is needed to determine the specific role of carbohydrates in MAE or other types of microalgae co-products on diet palatability and intestinal nutrient sensing mechanisms, both of which can affect feed intake of young pigs (Roura et al., 2016).

The fact that we observed similar feed intake and growth rate of pigs fed the 20% MAE diet and those fed the corn-soybean meal diet is encouraging, and suggests that MAE may be used to replace up to 20% of corn in diets for pigs without reducing growth performance if MAE is cost competitive with corn. In spite using a calculated value for

ME, our results suggest that the estimates calculated using the NRC equation were reasonably accurate for feeding pigs at 10 and 20% inclusion (Furbeyre et al., 2016). However, experimentally derived ME values are necessary for including MAE at greater than 20% inclusion or for routine and wide use of microalgae in pig feeding programs because variation among sources is likely to affect ME as observed for other biofuel coproducts (Urriola et al., 2014). The NRC equation that calculated ME using NDF as measure of non-starch polysaccharides will underestimate ME from MAE because a large proportion of polysaccharides in MAE are soluble. Likewise, soluble polysaccharides from MAE may have impacts on transit time, mucin production, and other physiology parameters that are not considered in the current estimates of ME. Therefore, experimental derived ME values for MAE are necessary.

Feeding diets containing up to 20% of the MAE may have beneficial environmental impacts by decreasing the carbon footprint of animal production as observed with other alternative feed ingredients fed to swine (Brune et al., 2009). This will become more important as more commercial pork production systems develop improved supply chain management of acquiring and using feed ingredients with a reduced carbon footprint (Mackenzie et al., 2016).

During this experiment, pigs developed signs of poor health and were treated with antibiotics in drinking water as well as injectable antibiotics for individual pigs. All groups of pigs fed MAE diets tended to have lower incidence of mortality and the number of pigs that required additional treatment was lower than those fed the control diet. These observations suggest that the MAE may have a health promoting effect in nursery pigs that deserves further exploration. Dietary supplementation of microalgae has

been studied for their health promoting potential, especially in relation to gut morphology and integrity. Furbeyre et al. (2017) studied the effects of microalgae species of the genera *Spirulina* and *Chlorella* on intestinal development and management of digestive disorders post-weaning in pigs. They observed increases in villi height in the jejunum of piglets fed diets supplemented with either 1% *Spirulina* or 1% *Chlorella*, suggesting a positive effect on mucosal restoration and development after weaning. Similarly, Dvir et al., (2000) fed rats a polysaccharide derived from *Porphyridium* and observed an increase in the number of goblet cells in the small intestine. We found no differences in mucosal height or goblet cells among dietary treatments, suggesting that the MAE co-product evaluated in the current study does not affect intestinal morphology. Likewise, we observed differences in both growth performance and health status of pigs fed the 1% and 5% MAE supplemented diets. A possible explanation of this effect is that microalgae carbohydrates can have prebiotic effects, promoting beneficial microbiota and production of short-chain fatty acids that favor improved growth performance and health status in pigs (De Jesus Raposo et al., 2016). Further studies are necessary to test the potential health benefits of MAE in diets for nursery pigs.

We also conducted a liver metabolomic analysis to determine if feeding MAE to nursery pigs would have beneficial metabolic effects. The analyses of hepatic metabolome and free amino acid concentrations revealed that MAE inclusion affects the concentrations of selected amino acid metabolites, including alanine, ammonia, arginine, histidine, and ornithine. Because ammonia, arginine, and ornithine are involved in the urea cycle, the decreased concentration of arginine and ornithine suggests that the urea cycle may be downregulated by feeding MAE, which may have led to increasing ammonia

concentration in the liver. It is well known that feeding fermentable carbohydrates such as inulin, soybean hulls, and sugar beet pulp to pigs can shift metabolism of amino acids from urinary N to microbial N excreted in feces (Aarnink and Verstegen, 2007). The carbohydrates in the MAE may serve as a source of fermentable cellulose or resistant starch capable of shifting ammonia excretion to the large intestine of pigs, which was observed by the changes in amino acid pattern in liver that is related to the urea cycle (Jha and Berrocoso, 2016).

In a recent metabolomic study in mice, Ma et al., (2015) observed that inclusion of 5% green algae in feed increased the ratio of glutathione (GSH) to oxidized glutathione (GSSG), while inclusion level of 20% algae decreased this ratio. In the present study, levels of GSH and GSSG were not affected by dietary inclusion of MAE (data not shown). However, the MAE co-product used in this study had a considerably lower concentration of CP than many algae preparations previously reported (Becker, 2004). Furthermore, it is probable that the lipid extraction process used to produce the MAE evaluated in this study, may have removed other nutrients which decreased its capability in altering redox balance or oxidative stress compared with other microalgae sources.

In conclusion, microalgae have the potential to serve as an environmentally sustainable nutrient source in animal production systems and may provide beneficial health effects. However, little is known about the benefits of specific fractions from microalgae on animal nutrition and health. The MAE co-product evaluated in this study had adequate energy and nutritional value to support optimal growth of pigs when included in diets at a 20% inclusion rate. Furthermore, it was interesting to note that this source of MAE may have potential health benefits to nursery pigs when added to diets at low inclusion rates.

However, repeatability of these benefits under different health conditions needs to be evaluated in future studies.

Table 2.1 Nutrient composition of the partially de-oiled microalgae extract (MAE) supplemented to nursery pig diets.

Nutrient	Value[1]
Proximate analysis, % as-fed	
Moisture	3.99
Crude protein	5.72
Ether extract	7.63
Ash	6.20
Carbohydrates by difference[2]	76.62
Total dietary fiber, %	33.30
Soluble dietary fiber	13.60
Insoluble dietary fiber	19.70
Neutral detergent fiber, %	7.70
Acid detergent fiber, %	2.87
Metabolizable energy content, kcal/kg[3]	2,600
Amino acid profile, % as-fed	**4.38**
Methionine	0.08
Cystine	0.07
Lysine	0.05
Phenylalanine	0.21
Leucine	0.42
Isoleucine	0.19
Threonine	0.20
Valine	0.29
Histidine	0.10
Arginine	0.13
Glycine	0.28
Aspartic acid	0.45
Serine	0.27
Glutamic acid	0.84
Proline	0.26
Hydroxyproline	0.02
Alanine	0.34
Tyrosine	0.10
Tryptophan	0.05
Taurine	0.03
Fatty acid profile, % as-fed	**7.19**
Capric	0.02
Lauric	0.08
Myristic	0.07

Palmitic	0.41
Palmitoleic	0.01
Heptadecanoic	0.00
Stearic	0.20
Oleic	6.20
Linoleic	0.13
Linolenic	0.02
Arachidic	0.03
Eicosanoic	0.01
Eicosapentaenoic (EPA)	0.01
Glycosyl composition of non-hydrolyzed MAE[4]	
Arabinose	not detected
Rhamnose	not detected
Xylose	not detected
Mannose	11.2
Galactose	2.8
Glucose	86.0
Glycosyl composition of hydrolyzed MAE[5]	
Arabinose	2.3
Rhamnose	not detected
Fructose	not detected
Xylose	0.5
Mannose	10.8
Galactose	15.1
Glucose	71.3

[1]Values are the result of one analyzed sample.
[2]Calculated as 100 - (DM + CP + Ether Extract + Ash).
[3]Calculated (Noblet and Perez, 1993): *ME (kcal/kg as-fed) = 4,194 – (9.2 × ash) + (1.0 × CP) + (4.1 × EE) – (3.5 × NDF),* where CP, EE, and NDF composition data (g/kg) were used on an as-fed basis.
[4]Glycosyl composition (mol % as fed) before hydrolysis with 2 M trifluoracetic acid.
[5]Glycosyl composition (mol % as fed) after hydrolysis with 2 M trifluoracetic acid.

Table 2.2 Ingredient and nutrient composition of experimental diets (as-fed) fed during d 1 and 14 (phase 1)

Ingredient	Control	1%	5%	10%	20%
Microalgae extract	0.00	1.00	5.00	10.00	20.00
Corn	33.37	32.17	27.36	21.35	9.32
Lactose	20.00	20.00	20.00	20.00	20.00
Soybean meal, 47.5% CP	15.00	15.00	15.00	15.00	15.00
Soy protein concentrate	15.00	15.00	15.00	15.00	15.00
Whey, dried	5.50	5.50	5.50	5.50	5.50
Fish meal, menhaden	5.00	5.00	5.00	5.00	5.00
Soybean oil	3.06	3.26	4.05	5.04	7.01
L-Lysine HCl	0.18	0.19	0.20	0.21	0.24
L- Threonine	0.01	0.01	0.01	0.01	0.02
DL-Methionine	0.13	0.13	0.14	0.16	0.18
Monocalcium phosphate	1.23	1.23	1.23	1.23	1.22
Limestone	0.62	0.62	0.62	0.62	0.61
Salt	0.40	0.40	0.40	0.40	0.40
Vitamin and trace mineral premix[1]	0.50	0.50	0.50	0.50	0.50
Total	100	100	100	100	100
Calculated nutrient composition					
ME, kcal/kg	3480	3480	3480	3480	3480
CP, %	23.59	23.55	23.39	23.19	22.78
NDF, %	5.87	6.14	7.19	8.51	11.14
Ether extract, %	5.78	6.01	6.91	8.04	10.30
Linoleic acid, %	2.39	2.46	2.78	3.18	3.97
P % (total)	0.77	0.77	0.77	0.77	0.77
P % (STTD)[2]	0.46	0.46	0.46	0.45	0.44
Ca, % (total)	0.86	0.86	0.86	0.86	0.86
Ca:P	1.12	1.12	1.12	1.12	1.12
Total Lys %	1.60	1.60	1.60	1.60	1.59
Lactose %	23.96	23.96	23.96	23.96	23.96
Standardized ileal digestible					
Lys, %	1.49	1.49	1.49	1.49	1.49
Lys:ME (g/Mcal/kg)	4.28	4.28	4.28	4.28	4.28
Met/Cys %	0.82	0.82	0.82	0.82	0.82
Thr %	0.87	0.87	0.87	0.87	0.87
Trp %	0.27	0.27	0.27	0.27	0.26
Analyzed Composition					
GE, kcal/kg	4101	4084	4095	4238	4381
CP, %	22.66	24.08	22.43	21.65	20.73
Ether extract, %	4.35	4.51	7.12	7.80	10.52
Ash, %	6.10	5.67	5.51	6.27	6.55
Moisture, %	7.68	7.30	7.28	6.58	5.16

[1]Premix supplied the following nutrients per kilogram of diet: 11,023 IU of vitamin A as retinyl acetate; 2,756 IU of vitamin D_3; 22 IU of vitamin E as dl-alpha tocopheryl acetate; 4.41 mg of vitamin K as menadione dimethylpyrimidinol bisulfite; 9.92 mg of riboflavin;

55.11 mg of niacin; 33.07 mg of pantothenic acid as d-calcium pantothenate; 992 mg of choline as choline chloride; 0.06 mg of vitamin B_{12}; 14.3 mg of pyridoxine; 1.65 mg of folic acid; 2.20 mg of thiamine; 0.33 mg of biotin; 2.20 mg of iodine as ethylenediamine dihydroiodide; 0.30 mg of selenium as sodium selenite; 299 mg of zinc as zinc sulfate; 299 mg of iron as ferrous sulfate; 19.8 mg of copper as copper sulfate; and 17.6 mg of manganese as manganese oxide.
[2]Standardized total tract digestibility.

Table 2.3 Ingredient and nutrient composition of experimental diets (as-fed) fed during d 15 to 28 (phase 2)

Ingredient	Control	1%	5%	10%	20%
Microalgae extract	0.00	1.00	5.00	10.00	20.00
Corn	45.33	44.62	40.16	34.14	22.06
Soybean meal, 47.5% CP	30.00	30.00	30.00	30.00	30.00
Whey, Dried	3.75	3.75	3.75	3.75	3.75
Soy Protein Concentrate	5.00	5.00	5.00	5.00	5.00
Lactose	10.00	10.00	10.00	10.00	10.00
Fish Meal, menhaden	2.50	2.50	2.50	2.50	2.50
Soybean Oil	0.00	0.00	0.65	1.64	3.65
L-Lysine HCl	0.13	0.13	0.14	0.15	0.18
DL-Methionine	0.06	0.07	0.07	0.09	0.11
Monocalcium phosphate	1.00	1.00	1.00	1.00	1.06
Limestone	1.32	1.04	0.84	0.83	0.80
Salt	0.40	0.40	0.40	0.40	0.40
Vitamin and trace mineral premix	0.50	0.50	0.50	0.50	0.50
Total	100	100	100	100	100
Calculated Nutrient Composition					
ME, kcal/kg	3300	3300	3300	3300	3300
CP, %	23.41	23.41	23.27	23.07	22.65
NDF, %	7.47	7.78	8.86	10.18	12.80
Ether extract, %	3.09	3.13	3.91	5.05	7.33
Linoleic acid, %	1.08	1.07	1.32	1.72	2.52
P % (total)	0.69	0.69	0.69	0.69	0.71
P % (STTD)[2]	0.37	0.37	0.36	0.36	0.36
Ca% (total)	0.97	0.86	0.78	0.78	0.78
Ca:P	1.41	1.25	1.13	1.13	1.10
Total Lys %	1.49	1.49	1.49	1.48	1.48
Lactose	12.70	12.70	12.70	12.70	12.70
Standardized Ileal Digestible					
Lys %	1.35	1.35	1.35	1.35	1.35
Lys:ME (g/Mcal/kg)	4.10	4.10	4.10	4.10	4.10
Met/Cys %	0.74	0.74	0.74	0.74	0.74
Thr %	0.80	0.80	0.80	0.80	0.80
Trp %	0.26	0.26	0.26	0.26	0.26
Analyzed Composition					
GE, kcal/kg	3916	3831	3955	4005	4171
CP, %	22.25	25.59	21.14	23.10	22.00
Ether extract, %	1.87	2.10	4.04	4.20	6.70
Ash, %	7.86	5.41	5.77	5.66	6.83
Moisture, %	10.40	11.38	9.54	9.46	7.68

[1]Premix supplied the following nutrients per kilogram of diet: 11,023 IU of vitamin A as retinyl acetate; 2,756 IU of vitamin D_3; 22 IU of vitamin E as dl-alpha tocopheryl acetate; 4.41 mg of vitamin K as menadione dimethylpyrimidinol bisulfite; 9.92 mg of riboflavin; 55.11 mg of niacin; 33.07 mg of pantothenic acid as d-calcium pantothenate; 992 mg of choline as choline chloride; 0.06 mg of vitamin B_{12}; 14.3 mg of pyridoxine; 1.65 mg of folic acid; 2.20 mg of thiamine; 0.33 mg of biotin; 2.20 mg of iodine as ethylenediamine dihydroiodide; 0.30 mg of selenium as sodium selenite; 299 mg of zinc as zinc sulfate; 299 mg of iron as ferrous sulfate; 19.8 mg of copper as copper sulfate; and 17.6 mg of manganese as manganese oxide.
[2]Standardized total tract digestibility.

Table 2.4 Ingredient and nutrient composition of experimental diets fed during d 29 to 42 post-weaning (phase 3)

Ingredient Composition	Control	1%	5%	10%	20%
Microalgae extract	0.00	1.00	5.00	10.00	20.00
Corn	66.10	65.05	60.25	54.26	42.16
Soybean meal, 47.5% CP	30.00	30.00	30.00	30.00	30.00
Soybean Oil	0.00	0.05	0.83	1.81	3.82
L-Lysine HCl	0.41	0.41	0.42	0.43	0.46
DL-Methionine	0.12	0.12	0.13	0.14	0.17
Monocalcium phosphate	1.36	1.36	1.38	1.41	1.47
Limestone	0.96	0.96	0.95	0.94	0.91
Salt	0.44	0.44	0.42	0.40	0.40
Vitamin and trace mineral premix	0.50	0.50	0.50	0.50	0.50
L-Threonine	0.11	0.11	0.11	0.12	0.12
Total	100	100	100	100	100
Calculated Nutrient Composition					
ME, kcal/kg	3300	3300	3300	3300	3300
CP, %	20.28	20.24	20.08	19.88	19.47
NDF, %	9.02	9.29	10.35	11.66	14.29
Fat, %	3.48	3.56	4.46	5.58	7.87
Linoleic acid, %	1.45	1.45	1.77	2.16	2.96
P % (total)	0.68	0.68	0.69	0.69	0.70
P % (STTD)[2]	0.35	0.36	0.36	0.37	0.37
Ca% (total)	0.72	0.72	0.72	0.72	0.72
Ca: P	1.06	1.06	1.04	1.04	1.03
Total Lys %	1.40	1.40	1.40	1.39	1.39
Standardized Ileal Digestible					
Lys %	1.27	1.27	1.27	1.27	1.27
Lys:ME (g/Mcal/kg)	3.85	3.85	3.85	3.85	3.85
Met/Cys %	0.70	0.70	0.70	0.70	0.70
Thr %	0.75	0.75	0.75	0.75	0.75
Trp %	0.21	0.21	0.21	0.21	0.21
Analyzed Composition					
GE, kcal/g	3885	3853	3897	3954	4039
CP, %	18.66	19.12	25.53	17.65	17.50
Ether extract, %	2.53	2.10	3.82	4.23	8.59
Crude fiber, %	3.37	2.28	2.59	2.89	3.15
Ash, %	5.09	5.43	6.15	5.50	5.70
Moisture, %	12.27	12.36	11.91	10.95	9.62

[1]Premix supplied the following nutrients per kilogram of diet: 11,023 IU of vitamin A as retinyl acetate; 2,756 IU of vitamin D_3; 22 IU of vitamin E as dl-alpha tocopheryl acetate; 4.41 mg of vitamin K as menadione dimethylpyrimidinol bisulfite; 9.92 mg of riboflavin; 55.11 mg of niacin; 33.07 mg of pantothenic acid as d-calcium pantothenate; 992 mg of choline as choline chloride; 0.06 mg of vitamin B_{12}; 14.3 mg of pyridoxine; 1.65 mg of folic acid; 2.20 mg of thiamine; 0.33 mg of biotin; 2.20 mg of iodine as ethylenediamine dihydroiodide; 0.30 mg of selenium as sodium selenite; 299 mg of zinc as zinc sulfate;

299 mg of iron as ferrous sulfate; 19.8 mg of copper as copper sulfate; and 17.6 mg of manganese as manganese oxide.
[2]Standardized total tract digestibility.

Table 2.5 Effects of feeding partially de-oiled microalgae extract on growth performance of nursery pigs

Item	Control	1%	5%	10%	20%
Number of pens	12	12	12	12	12
BW, kg					
d 0	6.26	6.27	6.28	6.27	6.27
d 7[2]	7.00[b]	7.25[a]	7.18[a,b]	7.22[a]	7.02[b]
d 14[1,2]	9.03[b]	9.50[a]	9.64[a]	9.66[a]	8.81[b]
d 21[2]	12.73[b]	13.47[a]	13.58[a]	13.48[a]	12.73[b]
d 28[2]	16.44[b]	17.49[a]	17.74[a]	17.73[a]	17.06[a,b]
d 35[2]	21.62[y]	22.82[x]	22.15[x,y]	22.77[x]	21.74[y]
d 42[2]	26.26[y]	27.58[x]	27.46[x,y]	27.78[x]	26.54[y]
SEM			0.54		
P - value					
Period			< 0.01		
Diet			0.05		
Period × diet			< 0.01		
Final pen body weight, kg[2]	126[b]	136[a,b]	133[a,b]	138[a]	133[a,b]
ADG, g					
d 1-7	92[b]	123[a]	112[a,b]	119[a]	96[a,b]
d 8-14[2]	287[b,c]	322[a,b]	340[a,b]	349[a,b]	256[c]
d 15-21	528	565	552	546	561
d 22-28	548	585	596	607	618
d 29-35	734[a,b]	778[a]	621[c]	720[a,b]	669[b,c]
d 36-42	784	815	883	836	799
Overall	496[z]	531[x]	517[x,y,z]	529[x,y]	500[y,z]
SEM			28.6		
P - value					
Period			< 0.01		
Diet			0.02		
Period × diet			0.07		
ADFI, g					
d 1-7[2]	120[b]	147[a]	138[a]	139[a]	122[ab]
d 8-14[2]	372[c]	380[b,c]	409[a,b]	421[a]	358[c]
d 15-21	742	749	736	755	701
d 22-28	718[b]	838[a]	860[a]	835[a]	799[a]
d 29-35	1,067	1,106	1,025	1,046	1,017
d 36-42	1,371	1,377	1,353	1,367	1,313
Overall[1,2]	732[c]	766[a]	754[a,b]	760[b,a]	718[c]
SEM			30.8		
P - value					
Period			< 0.01		
Diet			0.11		
Period × diet			0.04		
G:F, g/kg					
d 1-7	763	853	823	848	788

d 8-14	765	846	817	823	713
d 15-21[1]	719[b]	761[a,b]	753[a,b]	721[b]	803[a]
d 22-28	778	704	695	735	780
d 29-35	689[a]	710[b]	600[a]	688[a]	658[a,b]
d 36-42	573	590	658	613	611
Overall	715	744	724	738	725
SEM			39.3		
P - value					
Period			< 0.01		
Diet			0.36		
Period × diet			0.06		

[1]Linear effect of microalgae extract inclusion ($P < 0.05$).
[2]Quadratic effect of microalgae extract inclusion ($P < 0.05$).
[a,b,c]Values in the same row with different superscript differ ($P < 0.05$).
[x,y,z]Values in the same row with different superscripts tend to differ ($0.05 < P$ 0.10).

Table 2.6 Concentrations of free amino acids in the liver of pigs fed control and diets supplemented with partially de-oiled microalgae extract[1]

Amino acids	Control	1%	5%	10%	20%
Alanine	2,378 ±323[ab]	2,158 ± 371[ab]	2,187± 347[ab]	1936 ±753[a]	2966 ± 484[b]
Arginine	128 ± 51[a]	72 ± 29[ab]	87 ± 32[ab]	53 ± 25[b]	60 ± 21[b]
Asparagine	614 ± 70	630 ± 220	541 ± 147	448 ± 162	617 ± 171
Aspartic acid	5,202 ± 1,486	3,511 ± 1,360	3,364 ± 1,047	3,896 ± 2,757	5,959 ± 1,916
Citrulline	39 ± 20	20 ± 25	36 ± 33	29 ± 29	46 ± 33
Glutamic acid	2,5068 ± 5,559	17,504 ± 7,277	17,454 ± 8,369	18,171 ± 11,312	28,990 ± 6,612
Glutamine	2,529 ± 753	1,934 ± 575	2,261 ± 477	2,282 ± 1,018	3,034 ± 506
Glycine	3991 ± 860	3,968 ± 212	4,892 ± 1,008	4281 ± 810	5,077 ± 985
Histidine	451 ± 142[a]	291 ± 49[ab]	297 ± 50[ab]	259 ± 93[b]	304 ± 111[a]
Iso/Leucine	389 ± 85	394± 56	399 ± 44	312 ± 52	367 ± 90
Lysine	230 ± 56	164 ± 65	169 ± 58	135 ± 66	170 ± 71
Methionine	788 ± 265	922 ± 180	966 ± 286	640 ± 388	885 ± 296
Ornithine	193 ± 74[a]	123 ± 79[ab]	131 ± 57[ab]	77 ± 58[b]	120 ± 58[a]
Phenylalanine	93 ± 12	100 ± 21	92 ± 15	74 ± 12	89 ± 20
Proline	146 ± 33	132 ± 23	145 ± 11	126 ± 20	127 ± 30
Serine	1,598 ± 666	1,463 ± 266	1,837 ± 518	1,527 ± 657	1,953 ± 427
Taurine	1,429 ± 503	880 ± 326	1189 ± 361	861 ± 430	941 ± 308
Threonine	407 ± 58	340 ± 85	334 ± 102	310 ± 134	509 ± 177
Tryptophan	6.75 ± 3.77	4.88 ± 4.37	4.50 ± 2.76	4.88 ± 3.31	6.68 ± 3.31
Tyrosine	152 ± 93	90 ± 63	86 ± 70	105 ± 46	73 ± 38
Valine	500 ± 85	504 ± 75	508 ± 52	393 ± 89	489 ± 87
Total AA	46,332 ± 8,397	35,204 ± 9,326	36,978 ± 10,586	35,919 ± 1,7295	52,773 ± 10,021

[1]Data presented are mean ± SD as µmol/g of tissue on an as is basis.
[a,b]Values in the same row with different superscript differ ($P < 0.05$).

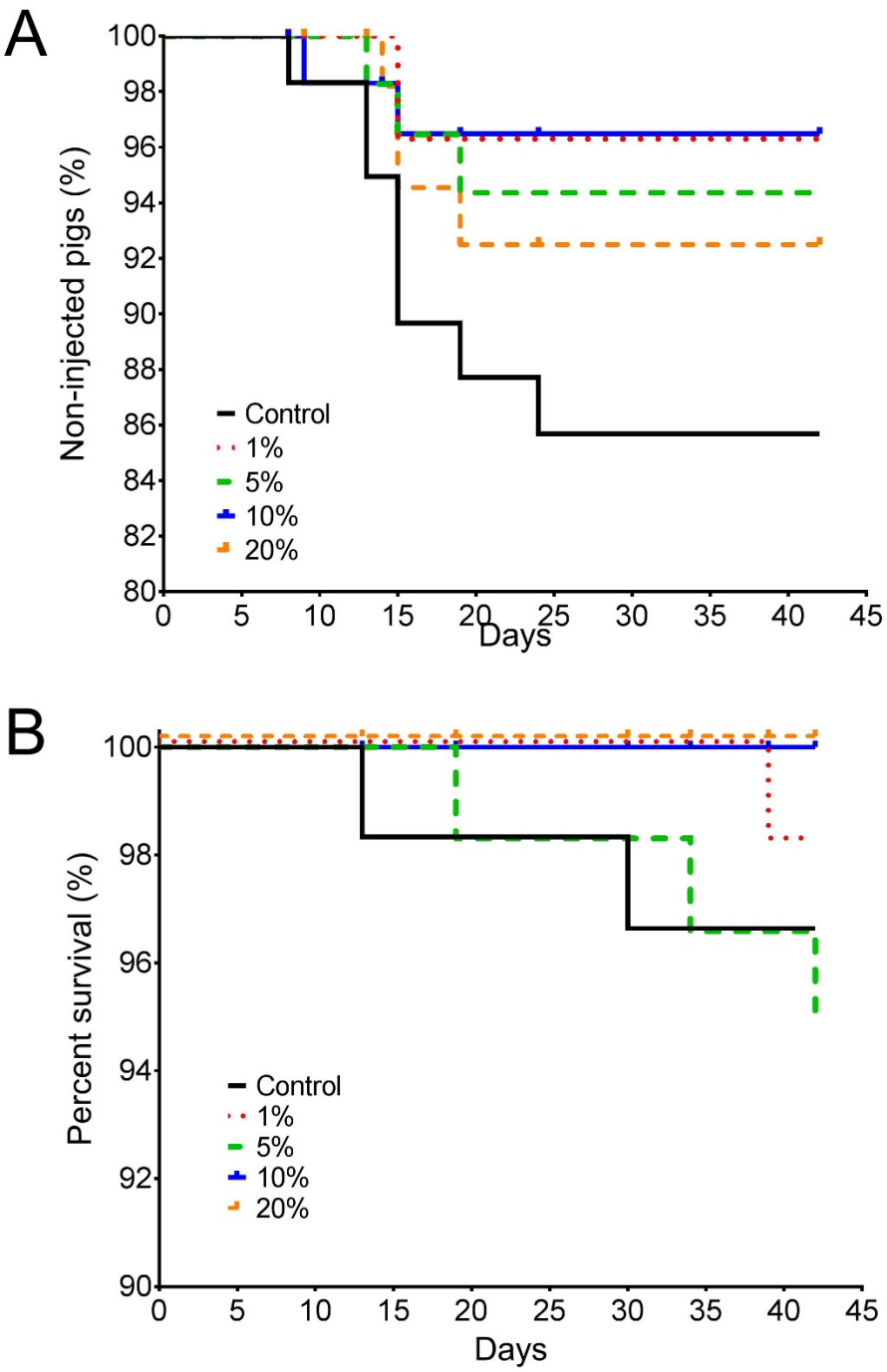

Figure 1 Antibiotic treatment and mortality in pigs fed diets supplemented with partially de-oiled microalgae product versus control diet. All pigs received antibiotic treatment in the drinking water from d 15 - 21, with some animals requiring additional enrofloxacin injection. (A) The magnitude of the drop at each day represents the percentage of pigs within a group that received enrofloxacin ($P = 0.16$). (B) Mortality of pigs fed diets with different amounts of partially de-oiled microalgae product ($P = 0.14$).

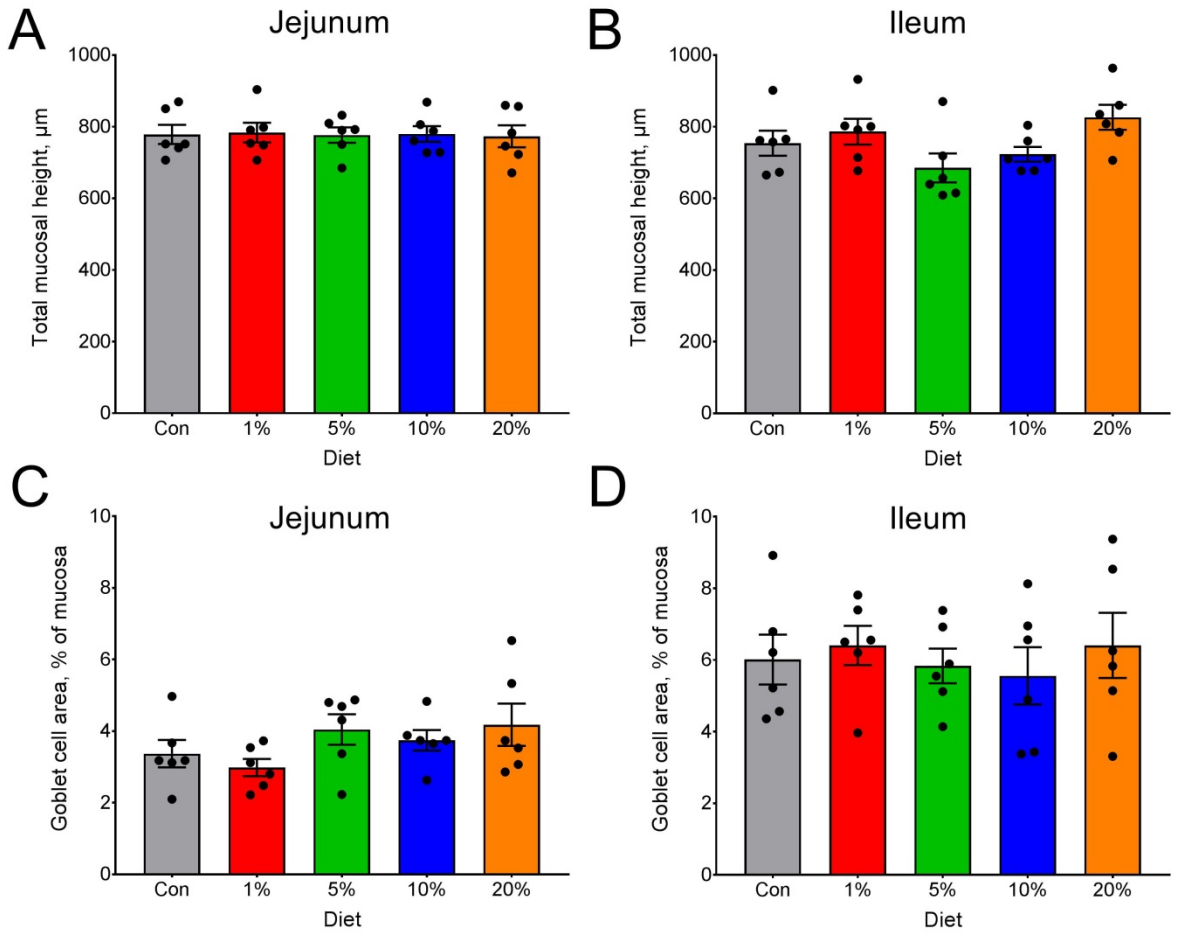

Figure 2 Histological analysis of jejunum and ileum in pigs fed diets supplemented with partially de-oiled microalgae product versus control diet. Mucosal height of the jejunum (A) and ileum (B) of pigs fed diets supplemented with partially de-oiled microalgae product. Quantification of mucin producing (Goblet) cells in jejunum (C) and ileum (D) of pigs fed the same diets. Bars heights are mean ± SEM (n = 6 pigs/group).

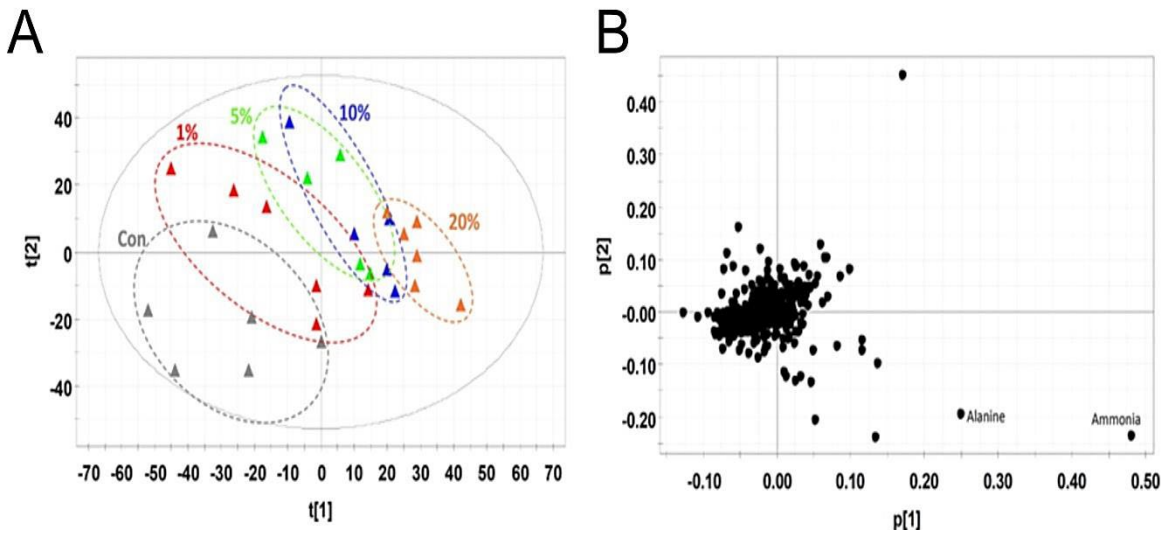

Figure 3 Effects of feeding partially de-oiled microalgae extract on the hepatic metabolome. (A) Score plot of a PCA model on the hepatic metabolome. The aqueous extracts of liver were derivatized by dansylation reaction to facilitate the detection of amino acids by LC-MS analysis. The t[1] and t[2] are the projection values of each sample in the 1st and 2nd principal components of the model, respectively. (B) Loading plot of a PCA model on the hepatic metabolome. The correlations of individual ions with the first and second components of the PCA model were indicated by their respective p[1] and p[2] values.

Chapter 3. Overall summary

With the demand for corn and soybean increasing due to an increase in world population and pork demand, swine producers are now challenged with researching alternative feed ingredients. The primary goals in using alternative feed ingredients in the swine industry are to reduce feed cost while maintaining growth performance in pigs, and to reduce the environmental impact with respect to overuse of natural resources and antibiotics to produce pigs. Previous literature and experiments described in chapter 1 suggest that microalgae have the potential to meet those goals for swine production systems.

The aim of the experiment described in chapter 2 was to investigate the use of a partially de-oiled microalgae extract as an alternative feed ingredient with a focus on promoting growth and health in nursery pigs. The nutritional composition of the MAE suggested that it could act as a health promoter, specifically as a prebiotic due to high levels of both digestible and potentially fermentable carbohydrates. For this reason, the MAE was included at low inclusion rates (1%, 5%). The high inclusion rates of the MAE (10%, 20%) were incorporated to determine its feeding value as an energy source in nursery pig diets.

Results from chapter 2 showed that the greatest ADFI and ADG were observed in nursery pigs fed the 1% and 10% inclusion of MAE compared to the corn-soybean meal diet. Additionally, pigs fed 1% and 10% MAE tended to require less individual treatments of antibiotics compared to the control to combat an unforeseen health challenge during experimentation. Gut morphology and metabolomics data were analyzed to explain the potential mechanisms by which the MAE increased both growth performance and health status in the nursery pigs. No significant differences were found in gut morphology of

pigs fed the control or MAE diets. Principal component analysis of liver amino acid metabolites revealed a dose-dependent separation of CON and MAE dietary treatments. Decreases in ammonia, arginine and ornithine levels were observed from pigs fed 1% and 10%, suggesting a down-regulation of the urea cycle. High inclusion of the MAE led to difficulties in mixing of the diets at the start of the experiment, leading to poor flowability of diets within the pig feeders. Therefore, the angle of repose of each diet was analyzed to determine the overall flowability of the MAE in nursery pig feeders. Results showed that as the inclusion of the MAE increased, the angle of repose and flowability decreased, especially with the 10% and 20% diets. Overall, these data suggest that the MAE could be a viable alternative feed ingredient for nursery pigs, especially at 1% inclusion due to observed increases in growth performance and health status. In addition, care must be taken when incorporating the MAE at high inclusion rates to minimize additional maintenance needed to increase flowability in feeders.

3.1 Limitations and future directions

The first limitation that should be noted is that cost of the MAE was provided or analyzed, as it was a proprietary product. Therefore, the feasibility of incorporating the MAE in nursery pig diets from an economic viewpoint is not entirely known. A primary goal of using alternative feed ingredients is to decrease feed cost, as it represents 65-75% of swine production costs. Therefore, cost of the MAE must be known in the future in order to be successfully incorporated into swine production systems. Additionally, due to its proprietary nature, the specific species of microalgae used or the percentage of the product that was microalgae versus additional materials from its production was not

known in this experiment. Thus, any benefits shown in chapter 2 cannot be compared to other studies using microalgae in swine diets, as the nutritional composition varies greatly from species to species, and consequently the potential biological effects.

A prediction equation was used to calculate the ME of the MAE, which used the components of the MAE nutrient analysis including CP, EE, and NDF. These methods could have underestimated the energy value of the MAE. Therefore, future studies involving in vivo digestibility of ME should be conducted.

The second limitation of this study is the fact that the health challenge was not controlled, as pigs became ill during the later weeks of experimentation. In order to truly investigate the health promoting benefits of the MAE to act as a potential alternative to antibiotics and to promote repeatability of results, future controlled health challenge studies should be performed with the MAE.

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